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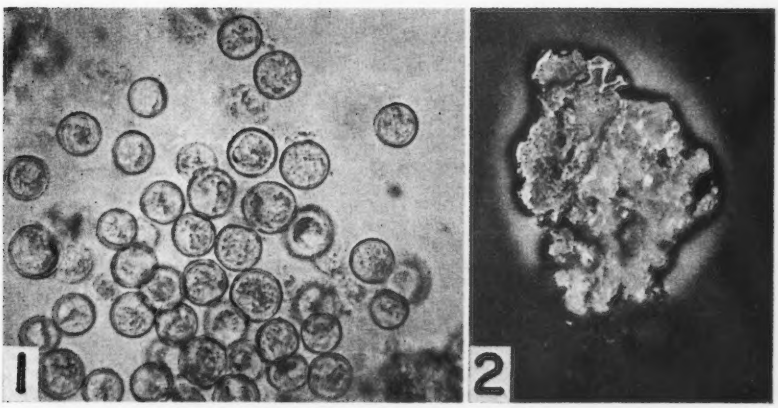

FIG. 1. Unstained amoebic cysts from a culture maintained on malt agar at 24° C. for 12 months, × 320.

FIG. 2. A 12-month-old culture of the free-living amoebae and Gram-negative bacilli maintained on malt agar at 24° C., × 1½.

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COMPLEMENT FIXING ANTIBODIES IN TUBERCULOUS GUINEA PIGS[1]

BY N. A. LABZOFFSKY, EVA KILLOUGH, W. J. A. PERCY, AND JAMES BELL

Abstract

Antigen prepared by a method previously described was employed in complement fixation tests on sera from guinea pigs experimentally infected with tuberculosis. In animals infected with a known inoculum of a single strain, complement fixing antibodies appeared regularly and consistently at the second to third week, reached a peak at about the seventh week, and thereafter either remained constant till the twelfth week, or declined. In another series sera from animals infected with unknown inocula of bacilli from a wide variety of sources all showed antibodies ranging in titer from 1:16 to 1:1024 at the sixth week.

Introduction

The results of experiments recently reported by Labzoffsky (1) indicate that in a limited number of sera from tuberculous human patients and guinea pigs complement fixing antibodies could be demonstrated regularly by the use of a new antigen derived from tubercle bacilli.

It was thought desirable to confirm this finding with respect to guinea pigs by performing similar experiments on a longer series, and also to determine the time after infection at which antibodies appear and the pattern of their development. These experiments are reported in the present communication.

Material and Methods

Antigen

The antigen was prepared from cultures of *Mycobacterium tuberculosis* strain H37Rv by the method previously described (1), which involved a preliminary pyridine extraction and then ultrasonic disruption of the cells in saline.

Sera

Two groups of guinea pig sera were employed in this study. The first group was from 43 guinea pigs of an average weight of about 300 gm. at the

[1]*Manuscript received March 22, 1956.*
Contribution from the Central Laboratory, Ontario Department of Health, 360 Christie Street, Toronto, Ont. This paper was presented in part at the twenty-third annual Christmas meeting of the Laboratory Section, Canadian Public Health Association, Toronto, Dec. 12 and 13, 1955.

[The June number of this Journal (Can. J. Microbiol. 2: 407-459. 1956) was issued May 31, 1956.]

time of inoculation, each of which had been inoculated intramuscularly in the thigh with 0.01 mgm. moist weight of living H37Rv tubercle bacilli. The animals were bled prior to inoculation and at weekly intervals thereafter for 12 weeks. Sera were separated from the clots and kept in the frozen state till the series was complete and were then tested simultaneously. The second group consisted of sera from 81 guinea pigs which had been infected with tuberculosis in the course of our routine diagnostic service. All the animals in this group were tuberculin positive six weeks after inoculation, all presented typical, bacteriologically proven, tuberculous lesions; but the infecting strains of bacilli were from a wide variety of sources: sputa, body fluids, and a few cultures of known tubercle bacilli. The number of bacilli in each inoculum was unknown. Each serum specimen in this group was obtained six weeks after the animal had been inoculated.

Complement Fixation Test

The technique of the test was that previously reported (2). The usual controls were always included with each test.

Tuberculin Tests

Six animals of the first group were skin tested with 1 mgm. of old tuberculin in 0.1 cc. at the second week after inoculation, six at the third week, and five at the fourth week. All animals of the second group were tuberculin tested at the sixth week.

Results

All preinoculation serum samples were negative. The results with sera after infection indicate that complement fixing antibodies appear regularly.

In the first group, i.e. sera from guinea pigs inoculated with a known inoculum of *M. tuberculosis* and bled at weekly intervals, antibodies were first demonstrable between the second and fourth week, reached a maximum by the seventh or eighth week, and thereafter fell into one of two distinct patterns.

The first type of response was characterized by the persistence of the maximum titer until the death of the animal or termination of the experiment. A typical example of this response is given in Table I. In the second type of response (Table II) the antibody level after reaching its maximum showed a gradual decline with progress of infection. In general this reduction in titer appeared to be associated with deterioration in the physical condition of the animal. All animals tuberculin tested gave positive reactions. The six tested two weeks after inoculation showed a positive tuberculin test whereas complement fixing antibodies were not demonstrable in these animals at that time. The maximum titers in this group ranged from 1:64 to 1:512.

In the second group, i.e. sera from guinea pigs inoculated six weeks previously with a variety of inocula, complement fixing antibodies were detectable in each case. The titers varied from 1:16 to 1:512 with one serum reaching a titer of 1:1024. The distribution of titers is shown in Table III.

TABLE I

ANTIBODY RESPONSE IN A GUINEA PIG INOCULATED WITH 0.01 MGM. OF LIVE TUBERCLE BACILLI

Serum, 1 in	8	16	32	64	128	256
Preinoculation	—	—	—			
1st week	—	—	—			
2nd "	4+	2+	—			
3rd "	4+	4+	4+	3+		
4th "	4+	4+	4+	4+	1+	
5th "	4+	4+	4+	4+	3+	
6th "	4+	4+	4+	4+	4+	1+
7th "	4+	4+	4+	4+	4+	2+
8th "	4+	4+	4+	4+	4+	3+
9th "	4+	4+	4+	4+	4+	3+
10th "	4+	4+	4+	4+	4+	3+
11th "	4+	4+	4+	4+	4+	3+
12th "	4+	4+	4+	4+	4+	3+

TABLE II

ANTIBODY RESPONSE IN A GUINEA PIG INOCULATED WITH 0.01 MGM. OF LIVE TUBERCLE BACILLI

Serum, 1 in	8	16	32	64	128	256
Preinoculation	—	—	—			
1st week	—	—	—			
2nd "	—	—	—			
3rd "	4+	4+	4+			
4th "	4+	4+	4+	2+		
5th "	4+	4+	4+	2+		
6th "	4+	4+	4+	3+	1+	—
7th "	4+	4+	4+	4+	3+	2+
8th "	4+	4+	4+	2+	1+	—
9th "	4+	4+	3+	1+	—	
10th "	4+	4+	2+	—	—	
11th "	4+	4+	2+	—	—	
12th "	4+	2+	—	—		

TABLE III

DISTRIBUTION OF C.F. TITERS OF SERA FROM 81 GUINEA PIGS INOCULATED SIX WEEKS PREVIOUSLY WITH ROUTINE T.B. SPECIMENS

C.f. titer, 1 in	8	16	32	64	128	256	512	1024
No. of sera	0	10	16	25	14	9	6	1

Comments

The results reported in this communication indicate that the complement fixing antibodies develop regularly in the sera of experimentally infected tuberculous guinea pigs and are demonstrable about the second or third week after infection. The antibody titer reaches its peak about the seventh or eighth week and then either persists or declines. As to the reason for these falls in titer one can only speculate. The impression that in general, though by no means consistently, the animals showing this drop in titer were in worse physical condition than the others suggests that the body defences, including antibody production, were becoming exhausted by overwhelming infection. It is of interest to note that patterns similar to both of these were observed in the sera of terminal cases of human tuberculosis. These are being investigated further and will be reported later.

In the second experiment guinea pigs were infected with strains of bacilli from a wide variety of sources, yet, in all, antibodies against antigen derived from one specific strain (H37Rv) were demonstrable. This would indicate that, although there may be antigenic differences between them, all had at least one antigenic component in common. Differences in titer may be accounted for by differences in antibody producing power of the individual animals, differences in size of inoculum, or possibly by antigenic differences of the infecting strains. The development of tuberculin sensitivity preceded by at least one week the appearance of complement fixing antibodies. These observations, of course, were made on a very limited number of animals and may not hold true in all cases. The results obtained here suggest that the complement fixation test might prove useful in evaluating the efficacy of different vaccines.

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1. LABZOFFSKY, N. A. Experiences with a new antigen for a complement fixation test in tuberculosis. Can. J. Microbiol. 1 : 794–798. 1955.
2. LABZOFFSKY, N. A. Rapid agglutination test as a possible aid in the laboratory diagnosis of ornithosis. J. Infectious Diseases, 79 : 96–100. 1946.

THE LABELLING OF PERTUSSIS VACCINE WITH IODINE-131[1]

By J. E. Logan and B. W. Griffiths

Abstract

The method of Francis, Mulligan, and Wormall for the labelling of proteins with iodine-131 has been modified for use with pertûssis vaccine. A vaccine suitably labelled for *in vivo* experiments has been produced as a result of this modification.

Introduction

The radioactive isotope, iodine-131 (I^{131}), has been used successfully for labelling microorganisms. Pearson *et al.* (9) labelled *Mycobacterium tuberculosis* and a number of fungi with I^{131}. The labelled material, after filtration and washing with sterile saline, was injected intravenously into dogs and its distribution followed by external monitoring and radioautography of tissues. These workers found that the distribution of I^{131}-labelled microorganisms was different from that of inorganic I^{131} administered to control animals. Sternberg *et al.* (11) prepared I^{131}-labelled BCG and studied its distribution in normal and immunized guinea pigs. A number of workers (2, 5, 6, 7) have used a metabolite of microorganisms such as phosphorus-32 as a labelling agent but the advantages of using a non-metabolite such as I^{131} for *in vivo* experiments have been clearly pointed out (11).

The present study dealing with the method developed in our laboratory for the labelling of killed suspensions of *Hemophilus pertussis* with I^{131} was undertaken to aid our search for improved methods for the assay of *H. pertussis* vaccine. Wide variation in response was noted in both our normal and immunized mice following intranasal challenge with live *H. pertussis*. To properly evaluate our assay procedures, it was necessary to determine whether these extreme differences in response were due to normal animal variation or to uneven passage and retention of organisms in the lungs. It was felt that this information could be obtained by the administration of I^{131}-labelled pertussis vaccine. In this work, no attempt was made to retain the antigenicity by employing a method of trace labelling (3). Instead, the object was to find a method of fully labelling the vaccine in such a way that a high specific activity would be obtained.

Methods

Commercial pertussis vaccine* was used throughout this investigation. Prior to iodination, the organisms were washed free of the medium in which they were suspended. The contents of ten 6 cc. ampoules were pooled and centrifuged for 10 min. at 10,800 r.p.m. in a No. 2 International Centrifuge.

[1]*Manuscript received March 27, 1956.*
Contribution from the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.
**Connaught Medical Research Laboratories of the University of Toronto.*

The packed organisms were washed four times with 0.9% sodium chloride and made up to a final volume of 10 ml. with 0.9% sodium chloride. The efficiency of this procedure was shown by the negligible amounts of nitrogen found in the final wash. The nitrogen determinations were made on samples that had been nesslerized following ashing with selenium dioxide – sulphuric acid mixture. Colorimetry of the nesslerized samples was carried out on the Lumetron photoelectric colorimeter using the 465 mμ filter. The concentration of organisms in the vaccine following washing was determined by opacity measurements at a wavelength of 350 mμ using the Beckman DU Spectrophotometer. The World Health Organization International Opacity Standard, a ground glass suspension prepared to simulate the turbidity of 10×10^9 *Hemophilus pertussis* organisms/ml., was used as reference standard.

The iodine-131* used in the investigation was obtained in the form of carrier-free sodium iodide in basic sodium bisulphite solution. For radioactivity measurement, all the samples containing I^{131} were pipetted onto planchettes and made alkaline with a drop of 10% NaOH. The addition of the sodium hydroxide prevented the loss of iodine by vaporization when the labelled preparations were being dried on a sample spinner under the infrared lamp. The counts of the dry iodinated material were recorded using the Nuclear Chicago Model DS-1 Scintillation counter and the Model 192 Ultrascaler. Corrections for the natural decay of the radioisotope were made in processing the experimental data.

Development of Methods

Prior to the development of the method of iodination that is described below, direct application of two existing methods was made. First, we followed Sternberg's method (11) for labelling live BCG organisms with I^{131}. This method was developed to allow for minimal radiation damage to the live organisms by high specific activities. In our work with pertussis vaccine, maintenance of the viability of organisms was not a consideration and since, in our hands, their method yielded a vaccine of low specific activity, it was considered impractical for these studies. Secondly, we used the method described by Francis, Mulligan, and Wormall (3) for the preparation of proteins labelled with I^{131}. In this method, free iodine is liberated from an "iodide–iodate" mixture with hydrochloric acid. The solution containing free iodine is then added to a suspension of the vaccine in ammonium hydroxide solution. This method, too, yielded a labelled vaccine of relatively low specific activity—11 to 12% of the radioactivity to which the vaccine was exposed being taken up. To obtain a labelled product of higher specific activity, the following factors were investigated: (1) relative concentrations of carrier potassium iodide and potassium iodate; (2) concentration of radioactive sodium iodide; (3) time of iodination; (4) concentration of microorganisms. To study the first three factors, concentrations of killed organisms sufficient to take up all the free iodine liberated were used.

**Supplied by Atomic Energy of Canada Limited, Commercial Products Division, Ottawa.*

(1) *Effect of Relative Concentrations of Carrier Potassium Iodide and Potassium Iodate*

One hundred microcuries of sodium iodide-131 were added to "iodide–iodate" mixtures of varying relative concentrations of carrier potassium iodide and potassium iodate. The potassium iodide/potassium iodate ratio ranged from 0.00 to 12.0. The free iodine was liberated from the "iodide–iodate" mixture by the addition of 0.2 ml. of 0.1 *N* hydrochloric acid. The iodine solution was added a few drops at a time to 2.0 ml. of the vaccine to which had been added 0.8 ml. of 0.5 *N*-ammonium hydroxide. Iodination was carried out for 15 min. at room temperature with continuous stirring. After iodination the labelled vaccine was freed of unbound iodine by the method of Sternberg *et al.* (11): washing with *M*/15 phosphate buffer, 1% potassium iodide, 1% sodium thiosulphate, and 0.1% sodium thiosulphate. The final water washes used in their studies were omitted, however, since we found it difficult to completely pack iodinated vaccine suspended in water. All centrifugations were carried out at 10,800 r.p.m. Negligible activity was recorded in the final wash. The iodinated vaccine was counted and results of the counts obtained expressed as percentage of the original count of I^{131} to which the material was exposed. The percentage uptake of labelled free iodine (I_2^{131}) by the vaccine for varying relative amounts of carrier potassium iodide and potassium iodate is shown in Table I. The greatest uptake of I_2^{131} by the vaccine was in the range of ratios from 0.72 to 2.89 of carrier potassium iodide/potassium iodate.

(2) *Effect of Radioisotope Concentration*

Varying amounts of sodium iodide-131 were added to an iodination mixture containing 240 μgm. carrier potassium iodide and 428 μgm. potassium iodate. No significant variation in the percentage uptake of I_2^{131} by the vaccine was

TABLE I

EFFECT OF RELATIVE CONCENTRATIONS OF CARRIER POTASSIUM IODIDE AND POTASSIUM IODATE ON THE UPTAKE OF I_2^{131} BY PERTUSSIS VACCINE

Carrier potassium iodide, μgm.	Potassium iodate, μgm.	Molar ratio carrier potassium iodide/ potassium iodate	% Uptake I_2^{131} by vaccine
0	428	0.00	1.2
30	428	0.09	7.7
80	428	0.24	17.9
120	428	0.36	18.1
240	428	0.72	22.6
400	428	1.20	21.3
700	428	2.10	21.8
960	428	2.89	21.8
415	107	5.00	18.9
500	107	6.02	16.6
840	107	10.1*	11.5
1000	107	12.0	11.0

**Relative concentrations used by Francis et al. (3).*

observed when the amount of radioisotope was varied between 40 and 500 microcuries (i.e. the total activity of the vaccine was proportional to the amount of sodium iodide-131 added).

(3) *Effect of Time of Iodination*

This factor was studied by continuous stirring of the vaccine throughout iodination periods of 15 min., one hour, and four hours. It was found that the uptake of I_2^{131} by the vaccine was not increased by exposing the organisms to periods of time longer than 15 min.

(4) *Effect of Concentration of Organisms in the Vaccine*

In studying the effect that varying the concentration of organisms has on the uptake of I_2^{131}, we used the optimal conditions of iodination as determined above. The concentrations of organisms were varied from 29×10^9 to 1166×10^9. These were iodinated with 100 μc. sodium iodide-131. The results are shown in Table II, where it will be seen that the output of I_2^{131} was only fully utilized when a concentration of 146×10^9 organisms or more was present in the vaccine or when the ratio of free iodine to the number of organisms $\times 10^{-9}$ was between 0.75 and 1.5.

Present Method of Iodination

As a result of these investigations, the following technique for iodination of pertussis vaccine was developed:

(a) Two milliliters of a suspension of pertussis vaccine containing approximately 146×10^9 organisms/ml. are centrifuged at 10,800 r.p.m. The supernatant is discarded.

(b) The packed cells are washed four times with 0.9% sodium chloride to free the vaccine of traces of medium, the same speed of centrifugation being maintained throughout.

(c) One hundred microcuries or more of the sodium iodide-131 are added (this amount is usually contained in about 50 μl. volume) to a mixture of 0.20 ml. of 0.12% potassium iodide (240 μgm.) and 1.2 ml. of 0.01 *N* potassium iodate (428 μgm.).

TABLE II

EFFECT OF CONCENTRATION OF ORGANISMS ON THE UPTAKE OF I_2^{131} BY PERTUSSIS VACCINE

Number of organisms $\times 10^{-9}$	Free iodine produced, μgm.	Free iodine/No. organisms $\times 10^{-9}$	% Uptake I_2^{131} by vaccine
29	220	7.59	4.7
58	220	3.79	11.8
87	220	2.53	14.4
117	220	1.88	18.2
146	220	1.51	21.4
292	220	0.75	21.2
1166	220	0.19	17.0

(d) A 0.8 ml. quantity of 0.5 *N* ammonium hydroxide is added to the vaccine which has been resuspended in 2.0 ml. of 0.9% sodium chloride.

(e) Free iodine is liberated from the iodide–iodate mixture by the addition of 0.2 ml. of 0.1 *N* hydrochloric acid.

(f) The solution containing the free iodine is added, a few drops at a time, to the ammoniacal solution of the vaccine.

(g) The vaccine is stirred continuously for a period of 15 min. at room temperature to ensure maximum iodination.

(h) The iodinated vaccine is centrifuged and the supernatant discarded.

(i) It is then washed twice with *M*/15 phosphate buffer, pH 7.4, twice with 1% potassium iodide, three times with 1% sodium thiosulphate, twice with 0.1% sodium thiosulphate (a total of nine washes), and the final wash is checked for radioactivity.

(j) The washed iodinated vaccine is resuspended in 0.9% sodium chloride to the volume desired for animal experimentation.

To test the precision of the above technique, replicate iodination experiments were carried out using the same concentration of organisms throughout. The statistical evaluation of these experiments is shown in Table III. I_2^{131} uptake by pertussis vaccine was 22–23% of the original amount of radioactivity to which the vaccine was exposed. This figure does not allow for loss of organisms in the washing procedure since the final count was taken after all washings were completed. The loss of the organisms by the iodination and subsequent washing is approximately 11% so that the actual uptake of I_2^{131} by the vaccine must have been between 24–25% of the total radioactivity to which it had been exposed.

TABLE III

UPTAKE OF I_2^{131} BY SUSPENSIONS OF PERTUSSIS VACCINE CONTAINING 146×10^9 ORGANISMS/ML.

Amount radioisotope, μc.	No. expts.	% Uptake I_2^{131} ± S.D. ± S.E.M.
100	8	22.6 ± 1.9 ± 0.72
200	3	23.0 ± 1.3 ± 0.93

Loss of Organisms Caused by Washings Prior to and Following Iodination

In our experience, the opacity measurements at 350 mμ of dilutions of *H. pertussis* suspensions varying between 2.3×10^9 and 14.5×10^9 organisms per ml. follow Beer's Law. To check the loss in the number of organisms in the vaccine prior to and following iodination, opacity readings were carried out on untreated vaccine, vaccine following washing to remove medium, and vaccine following iodination and the washing necessary to remove unbound iodine. The results are shown in Table IV. The total loss incurred by washing prior to iodination, the iodination process, and subsequent washing was approximately 21%.

TABLE IV

LOSS OF ORGANISMS FROM PERTUSSIS VACCINE BY WASHING PRIOR TO AND FOLLOWING IODINATION

Pertussis vaccine	No. organisms/ml. ampoule	% Loss
Unwashed	29.5×10^9	—
Washed	26.0×10^9	10.8
Iodinated	23.2×10^9	21.4

Attachment of I_2^{131} to the Pertussis Vaccine

To determine the mode of attachment of iodine to the vaccine, iodinated pertussis vaccine was sonically disintegrated in a 10 kc. Raytheon sonic oscillator using a 45 min. exposure time. The resulting material was fractionated into a trichloroacetic acid-soluble fraction, a lipid fraction, a nucleic acid fraction, and a residual protein fraction using Schneider's method (10). Our findings based on two separate experiments showed that the highest specific activity was associated with the protein fraction. In an attempt to locate the point of iodine attachment, the protein fraction was hydrolyzed in 6 *N* HCl at 110° C. for 16 hr. Paper chromatographic separation of the hydrolyzate components by the method of Pasieka, Morton, and Morgan (8) using a modified Woiwod's solvent system and a control chromatogram of tyrosine iodinated with I^{131} (4) indicated that the I^{131} activity was associated with a band probably due to 3,5-diiodotyrosine. This conclusion was supported by a positive Pauly reaction (1) and separation at the same distance from the origin for both chromatograms.

Discussion

Our results indicate that the chief factor in controlling the efficiency of labelling a predetermined amount of pertussis vaccine with I^{131} is the relative quantities of carrier iodide and potassium iodate present in the iodination system. Francis *et al.* (3) have suggested that the amount of carrier iodide should be kept to a minimum. In the iodide–iodate system, free iodine is liberated according to the equation:

$$5KI + KIO_3 + 6\,HCl = 6\,KCl + 3\,H_2O + 3\,I_2.$$

An examination of the relative amounts of potassium iodide and potassium iodate in a typical iodination of serum globulins shows that the above workers used 40 mgm. potassium iodide and 5.1 mgm. potassium iodate, i.e., a molar ratio of 10:1. The amount of potassium iodide required as calculated from the balanced equation would be 20 mgm. Francis *et al.* (3) have recommended a slight excess of potassium iodide, just sufficient to keep the liberated iodine in solution, for the iodination of the globulins.

In our investigation it has been found that an increase in the amount of carrier potassium iodide which would raise the molar ratio above the theoretical 5:1 figure lowers the uptake of I_2^{131} by the vaccine. In fact when the

molar ratio is increased to 10:1 as Francis *et al.* (3) have done the uptake is lowered by about one-half to a figure of 11.5 (Table I). Neglecting any effect of the low water solubility of free iodine, (see below) this result might be anticipated from a theoretical consideration of the system. The amount of potassium iodide has been raised to a figure double that which the potassium iodate present is capable of converting to free iodine. Therefore, only one-half of the sodium iodide-131 which becomes uniformly distributed throughout the carrier iodide is available for conversion to free iodine.

The optimum uptake of I_2^{131} by the vaccine in our system appears to occur when the molar ratio of potassium iodide/potassium iodate is 0.72 (Table I). The concentration of carrier potassium iodide in this system is 240 μgm. and the theoretical amount of free iodine generated is 220 μgm. It is unnecessary to have excess potassium iodide present to solubilize this small quantity of free iodine since 335 μgm. free iodine dissolve in 1 ml. water at 25° C. When the amount of free iodine produced exceeds the amount which will dissolve in the water present the percentage uptake of I_2^{131} falls off. The generation of a quantity of iodine in excess of its water solubility would appear to explain the decrease in uptake of I_2^{131} that we have observed when the theoretical iodide–iodate ratio of 5:1 is approached.

The conditions for iodination as outlined above constitute a more efficient use of the radioisotope than is possible with the method of Francis *et al.* (3) since the sodium iodide-131 is completely converted to free iodine. The total activity of the vaccine may be predetermined since it has been found that its activity is proportional to the amount of radioisotope used.

The effect of varying the concentration of organisms in the vaccine preparations has shown the minimum number of organisms (146×10^9) that must be present to utilize most efficiently the free iodine produced in our iodination system. Increasing the concentration of organisms in the vaccine eightfold lowered the percentage of uptake. This may have been due to a less efficient mixing of the iodine with the heavier suspension of organisms, since obviously there were more than enough organisms present to take up all the free iodine produced.

Sternberg *et al.* (11) found that the chemical washing procedure they employed for viable BCG organisms following iodination produced a loss of more than 80% of the initial number of microbes. Because of this high loss, they recommended a dialysis, in the presence of an anion exchanger, to remove the unfixed radioactive iodine. In this manner they reduced the loss below 10%. In our work with killed organisms, employing essentially the same washing procedure, the loss of organisms following iodination, when judged by opacity measurements, was approximately 10%, and the over-all loss, including the preliminary washing to remove traces of medium, did not exceed 22%. These figures are not comparable with those of Sternberg *et al.* (11) since their losses were assessed on a live bacterial count basis, whereas in the present investigation killed organisms were used and the losses were measured by opacity readings.

In a series of preliminary studies, we determined that the methods of Francis *et al.* (3) and Sternberg *et al.* (11) were not immediately applicable to the iodination of pertussis vaccine. A modified procedure was developed which, in our hands, proved more efficient than either of these methods for the labelling of pertussis vaccine with iodine-131.

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MORPHOLOGICAL AND PHYSIOLOGICAL GROUPS OF SOIL BACTERIA FROM THE ROOTS OF BARLEY AND OATS[1]

By H. DeL. King and R. H. Wallace[2]

Abstract

Morphological and physiological characteristics were studied of more than 2400 stock-cultures of bacteria isolated from rhizosphere soils of barley and oats, and from control soils. Gram-positive rods were proportionately more numerous in control soils than in rhizosphere soils; this difference was greater with oats than with barley. The proportion of Gram-negative rods was greater in rhizosphere soils than in controls but not more so for one crop than the other. Gram-positive rods replaced a significant portion of Gram-negative rods in soils of the mature barley plants. The only significant increase in percentage incidence of physiological groups in rhizosphere soils was in regard to nitrate-reducing bacteria of the young oats plants. On the other hand there were significantly smaller percentages for starch-hydrolyzing bacteria and gelatin liquefiers in oats rhizospheres than in the controls. There were not any significant differences between rhizospheres and controls with regard to physiological groups from the barley plants. It is concluded that the proportional incidences of some physiological groups of soil bacteria are not greatly increased, but in some instances are decreased, by the presence of barley or oat roots growing in Chicot sandy loam.

Fifty years ago Hiltner (5) discovered that the numbers of bacteria on or near roots of living plants are much greater than those in soil not in the immediate vicinity of roots; he used the term "rhizosphere" to designate that zone in which the plant-root seems to influence other physical and biological systems within its environment. Many investigators have confirmed Hiltner's discovery. Some have studied the kinds of bacteria from the rhizospheres of various crop-plants (6); a few, indeed, have observed that the equilibrium of morphological, physiological, or nutritional groups of soil bacteria seems to be noticeably altered in the rhizospheres of some plants but not in those of others (12, 13, 14). Most of these investigators, however, have made conclusions from data that either were not or could not be statistically analyzed for significant differences.

In estimating the total numbers of bacteria in a soil, it is preferable to prepare at least five replicate plates; the numbers of colonies on those plates must have a suitable distribution so that their mean will be an adequate numerical representation of the population under study (3). It may also be reasoned that the mean percentage incidence of a specific bacterial group in a given soil should be calculated from a number of percentage incidences, in a satisfactory number of plates, from a number of samples of that soil to obtain an adequate numerical representation of that group. A significant change in the proportional occurrence of a specific group may only be satisfactorily measured, therefore, by statistically comparing the data derived

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from as many samples as possible from each of two or more soils. For a complex experiment this may be done by means of an analysis of variance, and for a simple experiment by means of the *t* test. This principle was used in the investigation described in this paper. The object of the work was to isolate bacteria from the rhizospheres of field-grown barley and oats, and from control soils, to determine the distribution of morphological, physiological, and nutritional groups of those bacteria. The work was planned for the purpose of estimating significant changes that may be either generally characteristic of rhizosphere bacteria or related specifically to the types of plants or their ages. A report on methods and on the nutritional groups has been published elsewhere (12).

Experimental

Quantitative

Suitable dilutions of the soils were plated with soil extract agar (11) and colonies were counted for an estimation of the total numbers of bacteria and actinomycetes. The results are recorded in Table I, and the statistical data obtained from them are given in Table IV. It is to be noted that the numbers of microorganisms from the various samples were not significantly different, which suggests that the sampling and plating methods were sufficiently consistent, and that the microbial populations were numerically uniform. The great increase of numbers in the rhizosphere soils over those in the control soils confirms the results of many previous investigations and clearly demonstrates a quantitative "rhizosphere effect". The results also show that

TABLE I

Mean numbers of bacteria and actinomycetes from soils of two crops

(Millions/gm. of oven-dried soil)

	Barley		Oats	
Sample	Control	Rhizosphere	Control	Rhizosphere
Young				
1	18.49	77.90	4.09	240.30
2	22.35	260.10	4.57	264.20
3	16.47	169.20	4.19	361.60
4	15.57	245.40	3.12	137.67
5	16.28	353.00	4.17	265.70
Average	17.83	221.12	4.03	253.89
Mature				
1	9.46	60.84	6.50	47.40
2	8.83	48.92	9.52	92.04
3	8.82	57.57	7.48	89.83
4	9.65	72.89	6.57	52.11
5	7.52	138.60	7.61	117.64
Average	8.86	75.76	7.54	79.80

the increased population on the roots of the young plants was, in general, much greater than that of the mature plants. Other factors, such as kind of crop, and age of a specific crop, did not seem to affect the numbers of the population.

QUALITATIVE

Morphology

More than 2400 cultures were isolated and maintained in soil-extract semisolid agar for subsequent pure-culture studies. Transfers were made from the stock-cultures to tubes of a medium with the following composition: soil extract (8); K_2HPO_4, 0.02%; glucose, 0.01%; yeast extract, 0.01%; and agar 1.5%; the pH of the medium was adjusted to 6.8. The cultures were incubated at 25–28° C. for 48 hr. and then used for preparing films on slides. The films were stained by means of Hucker's modification of Gram's method, and the bacteria were grouped as Gram-positive and Gram-negative rods. Cultures with spherical cells were so few that they were not included in the analysis of results. In general the proportion of Gram-negative rods was distinctly greater than that of the Gram-positive rods, as found by many previous investigators (6). The percentages were transformed to arc sines (10) for statistical analysis because of the consistently large occurrence of the one group and of the consistently small occurrence of the other. The results are shown in Table II and the data obtained from the analysis of variance in Table IV.

There were smaller percentages of Gram-positive rods in the rhizosphere soils than in the controls; this difference seemed to be significantly greater in soil with oats than in that with barley. The percentages for Gram-negative rods, on the other hand, were greater in the rhizosphere soils than in the controls, yet not more so for one crop than the other. With regard to the age of the plants the proportions of Gram-positive rods in rhizosphere and control soils together were distinctly greater for mature barley than young barley; this was not so for oats. In contrast to this the proportions of Gram-negative rods were significantly greater in soils containing the young barley than in those with the mature plants; this also was not so for soils containing the oats. It seems, therefore, that the growth of the barley caused a general effect in that Gram-positive rods replaced a significant portion of the Gram-negative.

Physiology

Biochemical microtests described by Clarke and Cowan (2) were used to determine the ability of the respective bacteria to ferment glucose, hydrolyze starch, reduce nitrate, and liquefy gelatin. The bacteria were transferred to slopes of an agar medium similar to that used for the morphology studies; these were incubated at 25–28° C. for 48 hr. and a thick suspension of each was made in 2 ml. of tap water. The various quantities of suspensions recommended by Clarke and Cowan (2) were used for the respective microtests. The bacteria were grouped according to their physiological characteristics.

TABLE II

OCCURRENCE OF GRAM-POSITIVE AND GRAM-NEGATIVE RODS IN RHIZOSPHERE AND CONTROL SOILS OF TWO CROPS

	Barley								Oats							
	Young				Mature				Young				Mature			
	Control		Rhizosphere		Control		Rhizosphere		Control		Rhizosphere		Control		Rhizosphere	
Sample	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Gram-positive rods																
1	13/41*	31.70	4/61	6.55	18/57	31.57	3/66	4.54	18/59	30.50	7/65	10.76	17/71	23.94	3/71	4.22
2	5/32	15.62	2/69	2.89	22/60	36.66	4/44	9.09	20/67	29.85	7/62	11.29	24/73	32.87	3/73	4.10
3	15/62	24.19	6/66	9.09	19/51	37.25	5/62	8.06	30/71	42.25	7/69	10.14	18/62	29.03	5/71	7.04
4	8/58	13.79	8/55	14.54	11/63	17.46	6/67	8.95	18/60	30.00	6/65	9.23	18/60	30.00	17/73	9.58
5	13/50	26.00	6/67	8.95	17/61	27.86	14/70	20.00	33/67	49.25	5/72	6.94	20/48	41.66	10/74	13.51
Mean %		22.26		8.40		30.16		10.13		36.37		9.67		31.50		7.69
Gram-negative rods																
1	28/41	68.29	57/61	93.44	25/57	43.85	61/66	92.42	41/59	69.49	55/65	84.61	57/71	71.83	59/71	83.09
2	27/32	84.37	67/69	97.10	25/60	41.66	36/44	81.81	45/67	67.16	54/62	87.09	46/73	63.01	63/73	86.30
3	44/62	70.96	59/66	89.39	16/51	31.37	52/62	83.87	40/71	56.33	58/69	84.05	41/62	66.12	61/71	85.91
4	47/58	81.03	44/55	80.00	43/63	68.25	49/67	73.13	41/60	68.33	59/65	90.76	42/60	70.00	62/73	84.93
5	37/50	74.00	58/67	86.56	35/61	57.37	53/70	75.71	33/67	49.25	67/72	93.05	23/48	47.91	62/74	83.78
Mean %		75.73		89.29		48.50		81.39		62.11		87.91		63.77		84.80

**The figures on the right of the sloped lines are the numbers of cultures examined.*

As shown in Tables III, A, and IV the proportional occurrences of bacteria that formed acid from glucose seemed to be altered only in relation to the age of the barley plants; they increased considerably in soil containing the barley during the time that the plants grew from seedlings to mature plants, but not in the soil with oats. It is notable, however, that the proportions of these bacteria did not appear significantly different in control and rhizosphere soils. The results disagree, therefore, with those of previous investigations which showed that a larger percentage of rhizosphere bacteria ferment sugars than do those of control soils (1, 7).

The results given in Tables III, B, and IV show that the percentage incidences of the starch-hydrolyzing bacteria were greater in soil with the oats than in that of the barley. They also show that there was a significant reduction of these bacteria in the rhizosphere soils of oats, but not in those of barley. The age of the plants did not seem to be related to the occurrence of this group.

Tables III, C, and IV show that the proportional occurrences of nitrate-reducing bacteria were greater in the soils of the young plants than in those of the mature. This difference is attributable to changes in the control soils of barley and in the rhizosphere soils of oats. It is noteworthy that the various fluctuations of these bacteria masked the "rhizosphere effect" of the young oats; a *t* test of the control and rhizosphere data showed a significant difference between the two means; therefore the greater percentage occurrence of these bacteria was a specific characteristic of these rhizosphere soils. Differences between means from the other sets of data for control and rhizosphere soils were not significant.

TABLE III

OCCURRENCE OF BACTERIA THAT PRODUCE ACID FROM GLUCOSE, HYDROLYZE STARCH, REDUCE NITRATE, AND LIQUEFY GELATIN

	Barley				Oats			
	Control		Rhizosphere		Control		Rhizosphere	
Sample	No.	%	No.	%	No.	%	No.	%
A. *Bacteria that produce acid from glucose*								
Young								
1	17/41*	41.46	19/59	32.20	46/57	80.70	41/63	65.07
2	17/37	45.94	21/67	31.34	40/63	63.49	45/62	72.58
3	22/62	35.48	28/59	47.46	43/70	61.42	37/66	56.06
4	18/58	31.03	31/55	56.36	43/60	71.66	46/64	71.87
5	29/50	58.00	13/65	20.00	39/63	61.90	28/72	38.88
Mean %		42.38		37.47		67.83		60.89
Mature								
1	41/53	77.35	44/68	60.29	26/70	37.14	37/71	52.11
2	50/60	83.33	22/43	51.16	41/69	59.42	35/72	48.61
3	45/57	78.94	57/62	91.93	25/54	46.29	46/73	63.01
4	31/60	51.66	54/64	84.37	44/59	74.57	41/70	58.57
5	55/60	91.66	26/67	38.80	24/44	54.54	40/69	57.97
Mean %		76.59		65.31		54.39		56.05

TABLE III—(*Concluded*)

OCCURRENCE OF BACTERIA THAT PRODUCE ACID FROM GLUCOSE, HYDROLYZE STARCH, REDUCE NITRATE, AND LIQUEFY GELATIN

	Barley				Oats			
	Control		Rhizosphere		Control		Rhizosphere	
Sample	No.	%	No.	%	No.	%	No.	%
B. *Bacteria that hydrolyze starch*								
Young								
1	18	43.90	24	40.67	48	84.21	50	79.36
2	23	62.16	43	64.18	44	69.84	47	75.80
3	21	33.87	45	76.27	55	78.57	54	81.81
4	33	56.89	49	89.09	49	81.66	29	45.31
5	34	68.00	43	66.15	51	80.95	39	54.16
Mean %		52.96		67.27		79.04		67.28
Mature								
1	27	50.94	39	57.35	55	78.57	36	50.70
2	30	50.00	23	53.48	44	63.76	41	56.94
3	23	40.35	30	48.38	36	66.66	35	47.94
4	37	61.66	38	59.37	38	64.40	38	54.28
5	36	60.00	32	47.76	39	88.63	56	81.15
Mean %		52.59		53.27		72.40		58.20
C. *Bacteria that reduce nitrate*								
Young								
1	17	41.46	18	30.51	16	28.07	36	57.14
2	18	48.64	30	44.77	17	26.98	27	43.54
3	26	41.93	27	45.76	21	30.00	39	59.09
4	33	56.89	27	49.09	27	45.00	27	42.18
5	22	44.00	15	23.07	26	41.26	39	54.16
Mean %		46.58		38.64		34.26		51.22
Mature								
1	19	35.18	18	26.47	25	35.17	9	12.67
2	14	23.33	17	39.53	23	33.33	23	31.94
3	18	31.51	39	62.90	17	31.48	19	26.02
4	19	31.66	30	46.87	19	32.20	27	38.57
5	21	35.00	18	26.86	12	27.27	28	40.57
Mean %		31.34		40.53		31.89		29.95
D. *Bacteria that liquefy gelatin*								
Young								
1	29	70.73	41	69.49	45	78.94	29	46.03
2	30	81.08	55	82.09	54	85.71	40	64.51
3	34	54.83	40	67.79	53	75.71	40	60.60
4	43	74.13	32	58.18	42	70.00	49	76.56
5	36	72.00	45	69.23	49	77.77	41	56.94
Mean %		70.55		69.36		77.63		60.93
Mature								
1	40	74.07	32	47.05	50	71.42	22	30.98
2	46	76.66	17	39.53	45	65.21	28	38.88
3	40	70.17	28	45.16	42	77.77	18	24.65
4	41	68.33	37	57.81	44	74.57	27	38.57
5	49	81.66	34	50.74	33	75.00	30	43.47
Mean %		74.18		48.06		72.79		35.31

**The figures on the right of the sloped lines are the numbers of cultures tested. The same figures apply also to B, C, and D of this table.*

TABLE IV

ANALYSIS OF VARIANCE OF RESULTS

	Degrees of freedom	Numbers of bacteria and actinomyces		Morphological groups				Acid from glucose		Physiological groups				Gelatin liquefaction	
				Gram-positive		Gram-negative				Starch hydrolysis		Nitrate reduction			
		M.S.	F	M.S.	F	M.S.	F	M.S.	F	M.S.	F	M.S.	F	M.S.	F
Samples	4	3740.39	1.68	39.44	2.37	29.50	—	115.44	—	100.23	—	117.61	1.44	77.04	1.35
Age of plants	1	65980.69	29.77†	1.64	—	394.95	13.07†	1197.05	5.89*	566.48	3.57	855.71	10.51†	1447.44	25.49†
Soils	1	219284.26	98.94†	2544.02	152.88†	2522.85	83.53†	288.04	1.41	75.26	—	165.44	2.03	4151.40	73.12†
Crops	1	294.03	—	54.66	3.28	—	—	189.66	—	1615.82	10.19†	59.51	—	149.92	2.64
Age × soils	1	61613.86	27.80†	54.02	3.24	22.24	—	3.12	—	161.48	1.01	1.94	—	1305.76	23.00†
Age × crops	1	165.03	—	92.72	5.57*	287.24	9.51†	4032.46	19.87†	1.13	—	66.02	—	102.01	1.79
Soils × crops	1	1685.97	—	72.46	4.35*	—	—	74.36	—	1047.85	6.61*	118.64	1.45	451.04	7.94†
Age × soils × crops	1	1061.82	—	10.75	—	113.33	3.75	140.10	—	78.20	—	811.71	9.97†	10.69	—
Error	28	2216.29	—	16.64	—	30.20	—	202.94	—	158.45	—	81.37	—	56.77	—
Total	39	356042.34	—	2886.35	—	3400.31	—	6243.17	—	3804.90	—	2277.95	—	7752.07	—

**Significant.*
†Highly significant.

It is shown in Tables III, D, and IV that the percentage occurrences of gelatin-liquefying bacteria were greater in control soils than in rhizosphere soils, and that the difference was greater with regard to oats than with barley. Furthermore, the occurrences of these bacteria in the control and rhizosphere soils of young barley were about the same whereas they differed significantly in the control and rhizosphere soils of young oats; the latter effect was not evident from the analysis of variance but was brought out by the t test. The results are not in general agreement with those of Lochhead (7), who showed that a greater percentage of liquefying bacteria occurred in rhizosphere soils of tobacco and flax than in control soils; an exact comparison with Lochhead's work cannot be made, however, because of the different soils and crop-plants.

Discussion

As noted in a previous publication (12) the plants were field-grown in Chicot sandy loam. They were removed for sampling when they had grown to the "tiller" stage and later when they had matured to the "headed" stage. The time of seeding was arranged so that the laboratory work for the respective samples did not overlap. Samples were collected from rhizosphere soils and control soils at each of four sampling times, which were determined in accordance with the stages of growth of the plants. The average numbers of bacteria and actinomycetes, and the proportional incidences of specific groups of both the rhizosphere and control soils would be expected to fluctuate from one sampling time to another. Nevertheless, the relative data for rhizosphere and control soils at each sampling time may be compared, as well as those of one sampling time with another. The rhizosphere and control samples were collected at random along the rows of plants and between the rows, respectively. The statistical analysis of the results shows that there was not a significant difference between the respective samples, both in regard to numbers and specific groups; therefore the uniformity of the soil was established.

The results of many previous investigations of the physiological groups of soil bacteria appear to have been unsatisfactory for an understanding of the significance of those groups. Lochhead and Taylor (9), when reviewing the work of previous investigators, concluded that "the majority of soil bacteria are relatively inactive physiologically as judged by standard laboratory tests". It appears that more extensive experiments are required to give some understanding of the physiological potentialities of soil bacteria. Since it is shown in this investigation that the average incidences of three of the four groups were over 50%, it may be concluded that the soil bacteria are physiologically active. Recent work has also shown that the ability to produce acid from glucose is a predominant characteristic of Gram-negative short rods isolated from a variety of soils (4).

In microbiological studies of rhizosphere soils it is necessary to compare the findings with those obtained concurrently from control soils. Previous comparisons with physiological groups of microorganisms have shown a greater

percentage incidence of those groups in rhizosphere than in control soils (6). In this study, however, the only instance of a significant increase was in the percentage incidence of the nitrate-reducing bacteria of the young oat plants. In fact, some groups were significantly reduced within the rhizosphere: the starch-hydrolyzing bacteria and the gelatin liquefiers were proportionately fewer in rhizosphere soils of oats than in controls; these differences were greater in the mature plants than in the young.

Since the experiment was conducted only in one season with one soil-type, and but two cereal crops, the scope of generalization from the results is necessarily limited. The statistical analysis of data obtained from the various samples, however, leads to the conclusion that the proportional incidences of some physiological groups of soil bacteria are not greatly increased by the presence of barley or oat roots growing in Chicot sandy loam; indeed, those of some groups are noticeably decreased.

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A SEROLOGICAL STUDY OF PASTEURELLA HAEMOLYTICA[1]

By G. R. Carter

Abstract

It has been shown that strains of *Pasteurella haemolytica* possess a soluble capsular substance which is a polysaccharide. A haemagglutination test is described in which the soluble polysaccharide was adsorbed to type O human red cells. These treated red cells were agglutinated in the presence of appropriate dilutions of specific immune sera. Fifty-one strains of *P. haemolytica* isolated from cases of shipping fever in Canada were found to be serologically homogeneous by means of the haemagglutination test. Strains of *P. haemolytica* from the U.S.A., Great Britain, and Europe were found to be serologically identical with the Canadian strains by the haemagglutination and conventional agglutination test.

Introduction

It has been known for many years that *Pasteurella haemolytica* is an important pathogen of sheep and cattle. Recent observations indicate that this organism is the predominant bacterium in shipping fever in Canada (1, 3). This study was begun to determine whether or not the strains of *P. haemolytica* recovered from cattle with shipping fever were serologically homogeneous. This information would be necessary for the proper selection of cultures for the preparation of immunizing agents.

The organism *P. haemolytica* was first described by Jones (5) in 1921 and referred to as *P. boviseptica* Group I. The seven strains of *P. haemolytica* that he examined were serologically homogeneous and distinct from his Group II and III strains. These latter Groups were composed of strains now referred to as *P. multocida*.

Newsom and Cross (7) examined cultures of *Pasteurella* from sheep and cattle. They divided them into typical and atypical groups. Agglutination tests and biochemical reactions demonstrated that their typical group was made up of strains identical to Jones' Group I. The name *Pasteurella haemolytica* was proposed.

P. haemolytica has been frequently isolated from pneumonia and mastitis in sheep. Tunnicliff (9) referred to the organism recovered from cases of mastitis in ewes by himself and Marsh (6) as *Pasteurella mastiditis*. The strains of *P. haemolytica* recovered by Florent and Godbille (4) from pneumonia in sheep and cattle were found to fall into two groups serologically. Stamp and his colleagues (8) observed a septicaemia of lambs caused by *P. haemolytica*.

The inagglutinability of freshly isolated strains of *P. haemolytica* has been referred to by Tweed and Eddington (10). They pointed out that cultures became agglutinable after daily subculture for three weeks. Their nine strains of *P. haemolytica* were found to comprise the same serological type.

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Contribution from the Department of Pathology and Bacteriology, Ontario Veterinary College, Guelph, Canada.

A haemagglutination test in which the specific antigens were adsorbed on Type O human erythrocytes has been described for the typing of *P. multocida* (2). Polysaccharide antigens appear to be readily adsorbable on red cells whereas protein antigens appear to require alteration of the red cells.

Materials and Methods

Strains of P. haemolytica Examined

The 51 cultures including strains 1, 2, 3, 4, 5, and 6 were isolated from Canadian cattle with shipping fever. All of the strains referred to in two previous reports (1, 3) were included. In order to determine if strains of *P. haemolytica* from other parts of the world were identical to the Canadian strains some additional cultures were included. Details regarding these additional strains are listed in Table I.

Preparation of Immune Sera

Six immune sera, two prepared in rabbits and four prepared in calves, were employed. The immunizing procedure described previously for *P. multocida* was employed in the preparation of the rabbit sera (2). The schedule for the immunization of the calves is presented in Table II. Immune sera 1A, 1B, 1C, 1D, and 1E were prepared from strain No. 1. Serum 2A was prepared with strain No. 2.

TABLE I

DETAILS OF STRAINS OF *P. haemolytica* ISOLATED IN COUNTRIES OUTSIDE OF CANADA

Culture number	Geographic source	Animal origin	Disease
7, 8, 9, 10, 11, and 12	Montana, U.S.A.	Sheep	Mastitis
13 and 14	Scotland	Lambs	Septicemia
15 and 16	Belgium	Cattle	Pneumonia

TABLE II

SCHEDULE OF INJECTIONS FOR THE PREPARATION OF IMMUNE SERA IN CALVES

Day	Antigens: amounts in ml.		Route
	Dead*	Living	
1st	10	—	Subcutaneous
4th	10	—	Subcutaneous
6th	20	—	Subcutaneous
12th	20	—	Subcutaneous
20th	8	—	Subcutaneous
23rd	—	40	Intravenous

* *Killed by the addition of 0.25% formalin by volume.*

Preparation of the Capsular Substance

Two preparations of the capsular substance were obtained from each of the two strains 1 and 2. The organisms were cultivated overnight on fresh beef infusion agar contained in large (15 cm. diam.) Petri plates. The growth was washed off with cold distilled water, great care being taken to eliminate agar from the harvest of organisms. Each preparation consisted of capsular material of the organisms from 25 Petri plates. The suspensions of organisms in distilled water were heated in the water bath at 56° C. for 30 min. The bacterial cells were then removed by centrifugation.

The supernatant was lyophilized after which the capsular material obtained was redissolved in several milliliters of distilled water. After the solution had been centrifuged to remove insoluble material, it was lyophilized again. The dried capsular substance was a creamy color, flaky, and dissolved readily in distilled water and normal saline.

Preparation of Extracts of P. haemolytica

Saline extracts containing the capsular substances of all 51 strains, and the other strains listed above, were obtained in the manner described previously for *P. multocida* (2). These extracts containing the capsular substance were used for the treatment of the erythrocytes.

Treatment of Erythrocytes

The human type O red cells were prepared in the manner previously outlined (2). Two different preparations of capsular substances were employed for the treatment of erythrocytes. For the serological examination of all of the strains referred to above the capsular substances were employed as saline extracts. Treatment of the red cells was carried out as described previously (2).

In the tests, the results of which are presented in Table IV, the desiccated preparations of capsular substance were used to treat the red cells. The smallest amount of the desiccated material which gave the maximal haemagglutination titers was a 0.025% solution. To each 4 ml. of the solution of capsular substance there was added 0.2 ml. of packed erythrocytes. The mixture was incubated for two hours at 37° C. after which the cells were removed by centrifugation. The erythrocytes were washed once with 10 ml. of normal saline and then, after centrifugation, sufficient saline was added to give a 1% suspension.

Performance of the Haemagglutination Test

This test involved the addition of treated erythrocytes to dilutions of specific immune sera. A positive reaction consisted of clumping of the red cells. The procedure employed was identical with that described for typing *P. multocida* (2). In this previous report it was stated that the serum dilutions began at 1:10 and extended by doubling to 1:320. Actually these were the serum dilutions prior to the addition of treated erythrocytes. In this study the serum dilutions refer to the final dilutions. The controls listed in Tables III and IV refer to immune serum plus untreated erythrocytes.

TABLE III

HAEMAGGLUTINATION REACTIONS BETWEEN IMMUNE SERA AND HOMOLOGOUS ANTIGENS: ERYTHROCYTES TREATED WITH SOLUTIONS OF DESICCATED CAPSULAR SUBSTANCE

Immune sera	Capsular substance from strain No.	Reciprocals of serum dilutions					Controls
		20	40	80	160	320	
1A (rabbit)	1	+	+	+	+	±	−
1B (rabbit)	1	+	+	+	+	±	−
1C (calf)	1	−	−	−	−	−	−
1D (calf)	1	+	−	−	−	−	−
1E (calf)	1	+	+	±	−	−	−
2A (calf)	2	+	+	±	−	−	−

TABLE IV

TYPICAL HAEMAGGLUTINATION REACTIONS: ERYTHROCYTES TREATED WITH SALINE EXTRACTS OF THE STRAINS

Strain	Immune sera	Reciprocals of serum dilutions					Controls
		20	40	80	160	320	
1	1A	+	+	+	+	±	−
1	1B	+	+	+	+	±	−
2	2A	+	+	+	+	−	−
4	2A	+	+	+	−	−	−
5	2A	+	+	±	−	−	−

The tubes were left at room temperature for approximately two hours at which time the test was read. No purpose appeared to be served by a later reading.

Performance of the Agglutination Tests

Tweed and Eddington observed that freshly isolated cultures of *P. haemolytica* were frequently inagglutinable. All of the strains on which the agglutination tests were carried out had undergone a considerable number of transfers and were readily agglutinable.

Antigens for the agglutination test consisted of the overnight growth on sheep's blood agar. The harvested bacteria were washed once in saline, then diluted in 0.5 % phenolized saline to a turbidity corresponding to Brown's tube No. 2 (Burroughs, Wellcome and Co.).

Two-fold serial dilutions of the immune sera were prepared as for the haemagglutination test. To each tube containing 0.4 ml. of the serum dilution there was added 0.4 ml. of the suspension of bacterial cells. The control tube contained 0.4 ml. of saline and 0.4 ml. of the bacterial suspension. The tests were read in the conventional manner after incubation at 37° C. for 48 hr.

Results

Unhydrolyzed solutions of the four desiccated preparations of capsular material gave negative biuret tests and positive Molisch and Bial reactions. Reducing substances as determined in the Benedict test were liberated after hydrolysis with 2 *N* HCl in sealed ampoules in boiling water for three hours. These reactions demonstrated that the capsular substances were essentially carbohydrate in nature.

The haemagglutinating titers of the two rabbit sera and the four bovine sera are given in Table III.

The immune sera listed in Table III with the exception of No. 1C and 1D were employed in the serological examination of the 51 Canadian strains and the strains listed in Table I. Some typical results of the haemagglutination typing tests are given in Table IV.

The titers of all of the strains approximated those presented in Table IV. These results indicate that all of the strains examined represented one serological type.

For the purpose of confirming this serological homogeneity the conventional agglutination test was performed on representative strains of the whole collection of cultures. Reactions typical of those recorded are listed in Table V. They indicate again the serological homogeneity of the strains studied. Variation in titers may be related to the amount of capsular substance present on the organisms of a particular strain.

TABLE V

RESULTS OF AGGLUTINATION TESTS WITH *P. haemolytica*: STRAINS FROM DIFFERENT ANIMAL SPECIES AND COUNTRIES

Strain	Immune sera	Reciprocals of serum dilutions						Controls
		20	40	80	160	320	640	
5	1A	+	+	+	+	±	−	−
6	1A	+	+	±	−	−	−	−
8	1A	+	+	+	+	+	+	−
12	1A	+	+	+	+	+	+	−
13	1A	+	+	+	+	+	+	−
14	1A	+	+	+	+	+	+	−
15	1A	+	+	+	+	+	+	−
16	1A	+	+	+	+	+	±	−

Discussion

The efficacy of bacterins for the prevention of shipping fever in cattle has not been conclusively demonstrated. However, if bacterins continue to be employed in Canada for the prevention of shipping fever, *P. haemolytica* should be included along with the prevalent types of *P. multocida*.

It is of interest that although *P. haemolytica* is described as a haemolytic organism, a true haemolysin has not been demonstrated (11). Whether or

not 'haemolysis' of the red cells is caused by the diffusion into the medium of the capsular substance has not been determined. Woxholtt (11) has shown that antiserum inhibits the zone of 'haemolysis' but does not affect the growth of cultures.

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ENZYMES RELATED TO CARBOHYDRATE METABOLISM IN UREDOSPORES OF WHEAT STEM RUST[1]

By P. Shu and G. A. Ledingham

Abstract

Cell-free extracts of uredospores of wheat stem rust contained enzymes that with the addition of triphosphopyridine nucleotide can oxidize mannose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, and glucose-6-phosphate, to pentose-5-phosphates, and the latter are converted to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate through the transketolase reaction. Diphosphopyridine nucleotide linked α-glycerol phosphate dehydrogenase, triose isomerase, and aldolase activities were also detected. However, transaldolase activity was not found in the spore preparation. These findings support the idea that the glucose-6-phosphate oxidation pathway is actively involved in the carbohydrate metabolism of rust fungi.

In a previous study (10) it was observed that only protein and lipid but not the carbohydrates of the uredospores of wheat rust were utilized during the process of germination. The addition of a number of phosphorylated intermediates of carbohydrate metabolism did not effect the respiration of either the germinated or resting spores. From these observations, glycolytic enzymes were thought to be not actively involved in the process of germination. Nevertheless, radioactivity was detected in carbon dioxide produced by uredospores of wheat stem rust when supplied with labelled sugars (9), and glucose-6-phosphate dehydrogenase activity was found in spore material (1), definitely suggesting the presence of active enzyme systems related to carbohydrate metabolism in the spores.

This paper presents evidence which indicates the presence of the enzymes related to glucose-6-phosphate oxidation cycle in the uredospores of wheat stem rust.

Materials and Methods

Cell-free Enzyme Preparation from Uredospores of Wheat Stem Rust

Eight hundred milligrams of uredospores of wheat stem rust strain 15B were ground, in presence of 15 ml. of distilled water, in a planetary ball mill for 2.5 hr. at 1° C. Microscopic examination showed that the spores were completely ruptured. A clear cell-free extract was obtained by centrifuging the ground material twice, for 10-min. intervals each, in a Spinco centrifuge operated at av. 6500 *g* (10,000 r.p.m. with number 40 head).

Methods for Enzyme Detection

Methods used for the determination of enzyme activities were essentially the same as described in *Methods in Enzymology, Vol. I*, (2). Since this study was only concerned with the demonstration of the enzyme activity,

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the reactions were all carried out at room temperature. All the enzymes under investigation may be detected by observing the oxidation-reduction of pyridine nucleotides and the enzyme activities are, therefore, expressed in terms of change in optical density at 340 mμ using a Beckman Model DU Spectrophotometer.

The compositions of the reaction mixtures for various enzyme determinations are summarized below.

(1) Glucose-6-phosphate dehydrogenase

Basal	Glycylglycine buffer	0.2 *M* (pH 7.5)	0.2 ml.
	$MgCl_2$	0.1 *M*	0.1 ml.
	Triphosphopyridine nucleotide (TPN)	0.002 *M*	0.2 ml.
	Rust spore extract		0.2 ml.
Glucose-6-phosphate (G-6-P)		0.1 *M*	0.2 ml.
Distilled water		make up to total volume of	3.2 ml.

(2) 6-Phosphogluconate dehydrogenase

Basal as in (1)		
6-Phosphogluconate (6-P-G)	0.1 *M*	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

(3) Phosphoglucomutase

Basal as in (1)		
Glucose-1-phosphate (G-1-P)	0.1 *M*	0.2 ml.
Glucose-6-phosphate dehydrogenase	1 mgm./ml.	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

Yeast glucose-6-phosphate dehydrogenase (kindly supplied by Dr. E. R. Blakley of this laboratory) was prepared from yeast by the method of Kornberg (6).

(4) Phosphomannose isomerase

Basal as in (1)		
Mannose-6-phosphate (M-6-P)	0.1 *M*	0.2 ml.
Glucose-6-phosphate dehydrogenase	1 mgm./ml.	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

(5) Phosphoglucose isomerase

Basal as in (1)		
Fructose-6-phosphate (F-6-P)		0.2 ml.
Glucose-6-phosphate dehydrogenase	1 mgm./ml.	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

(6) Transketolase

Glycylglycine buffer	0.5 *M* (pH 7.5)	0.2 ml.
Dihydrodiphosphopyridine nucleotide (DPNH)	0.002 *M*	0.2 ml.

Triose isomerase and α-glycerol phosphate dehydrogenase 0.52–0.72 ammonium sulphate fraction of rabbit muscle extract prepared by the method of Racker (7)	10 mgm./ml.	0.1 ml.
"Yeast pentose phosphate isomerase product" prepared by the method of de la Haba and Racker (5)	3%	0.2 ml.
Thiamine pyrophosphate	0.1%	0.1 ml.
$MgCl_2$	0.1 *M*	0.1 ml.
Rust spore extract		0.5 ml.
Distilled water	make up to total volume of	3.2 ml.

(7) α-Glycerol phosphate dehydrogenase

Basal	Glycylglycine buffer	0.2 *M* (pH 7.5)	0.2 ml.
	DPNH	0.002 *M*	0.2 ml.
	Rust spore extract		0.1 ml.
Dihydroxyacetone phosphate (DAP)		0.1 *M*	0.2 ml.
Distilled water		make up to total volume of	3.2 ml.

(8) Triose phosphate isomerase

Basal as in (7)		
Glyceraldehyde-3-phosphate (G-3-P)	0.1 *M*	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

(9) Aldolase

Glycylglycine buffer	0.2 *M* (pH 7.5)	0.2 ml.
DPNH	0.002 *M*	0.2 ml.
Rust spore extract		0.2 ml.
Fructose-1,6-diphosphate (FDP)	0.1 *M*	0.2 ml.
Distilled water	make up to total volume of	3.2 ml.

Results and Discussion

The cell-free extract prepared from uredospores of wheat rust gave a clear, pale yellow solution. When fresh, it reduced TPN in presence of glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate but mannose-6-phosphate only slightly (Fig. 1). The spore extract lost its TPN reducing ability after four to five day's storage at $-10°$ C. However, with the addition of glucose-6-phosphate dehydrogenase, the aged extract was able to reduce TPN in presence of glucose-1-phosphate, fructose-6-phosphate, and even mannose-6-phosphate (Fig. 2). These results indicate clearly that the crude preparation contained a comparatively labile glucose-6-phosphate dehydrogenase. It also contained phosphoglucomutase and hexose-phosphate isomerases (which convert mannose-6-phosphate and fructose-6-phosphate to glucose-6-phosphate). An enzyme converting mannose-6-phosphate directly to glucose-6-phosphate

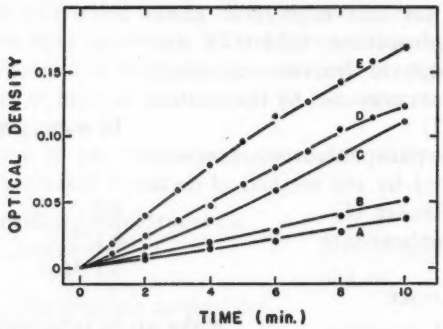


FIG. 1. Oxidation of hexose monophosphates by rust spore extract in presence of TPN. A, Basal; B, A + M-6-P; C, A + G-1-P; D, A + F-6-P; E, A + G-6-P.

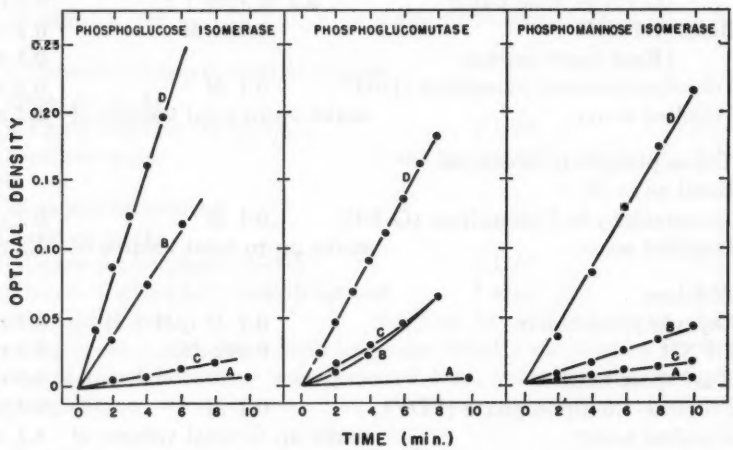


FIG. 2. Demonstration of phosphoglucose isomerase, phosphoglucomutase, and phosphomannose isomerase activities. A, complete less substrate; B, complete less spore extract; C, complete less glucose-6-phosphate dehydrogenase; D, complete.

has not yet been described in the literature, but under these experimental conditions, mannose-6-phosphate was probably converted to glucose-6-phosphate through the formation of fructose-6-phosphate. However, no attempt was made to verify this point.

As shown in Fig. 3, the spore extract demonstrated a strong 6-phosphogluconate dehydrogenase activity. This enzyme, in contrast to the glucose-6-phosphate dehydrogenase, was stable at 4° C. The presence of this enzyme was further confirmed by the observation of carbon dioxide evolution and ketose formation (4). Both the glucose-6-phosphate and 6-phosphogluconate dehydrogenases are TPN linked.

The crude preparation of rust spore enzymes oxidized DPNH in presence of "yeast pentose phosphate isomerase product". However, with added

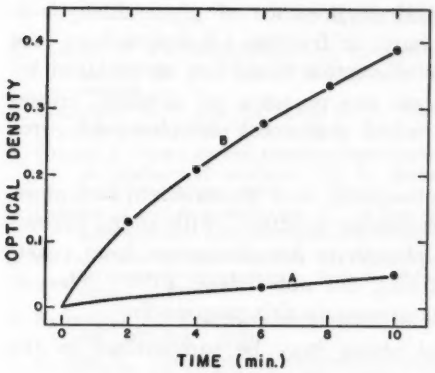


FIG. 3

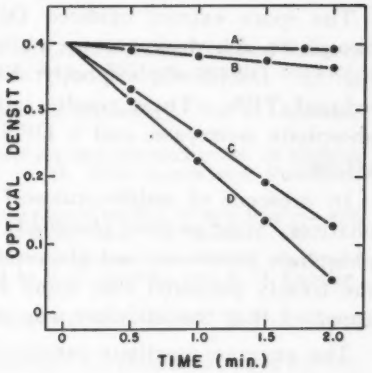


FIG. 4

FIG. 3. Demonstration of 6-phosphogluconate dehydrogenase activity. A, complete less substrate; B, complete.

FIG. 4. Demonstration of transketolase activity. A, complete less substrate; B, complete less spore extract; C, complete less α-glycerol phosphate dehydrogenase; D, complete.

α-glycerol phosphate dehydrogenase, the rate of oxidation was somewhat faster (see Fig. 4). After a longer period of incubation the reaction mixture gave a cysteine – sulphuric acid reaction similar to that for sedoheptulose (3). This indicated the presence of transketolase in the spore preparation. For further confirmation, hydroxypyruvic acid and ribose-5-phosphate were used as substrates. The formation of keto sugar was detected spectrophotometrically with the cysteine – sulphuric acid reaction (3) and the slow shift of absorption maximum from 392 mμ to 505 mμ resembled that of sedoheptulose.

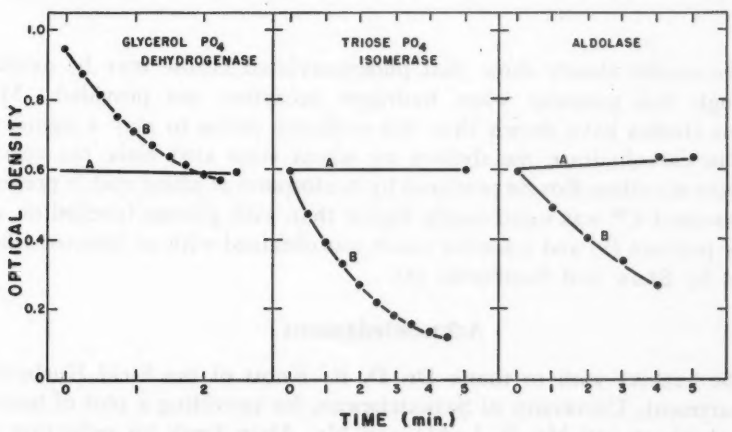
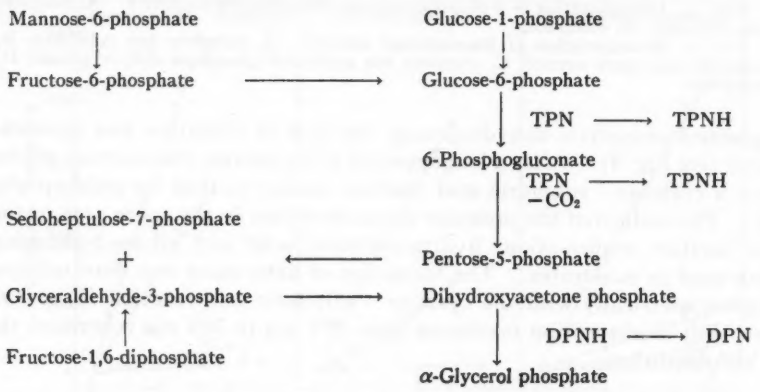


FIG. 5. Demonstration of α-glycerol phosphate dehydrogenase, triose phosphate isomerase, and aldolase activities. A, complete less substrate; B, complete.

The spore extract oxidized DPNH in presence of glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, or fructose-1,6-diphosphate (see Fig. 5). Dihydrodiphosphopyridine nucleotide could not be replaced by reduced TPN. These results indicate the presence of aldolase, triose-phosphate isomerase, and a DPN linked α-glycerol phosphate dehydrogenase.

In presence of sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate or "yeast pentose phosphate isomerase product", with added hexose-phosphate isomerase and glucose-6-phosphate dehydrogenase from yeast, the freshly prepared rust spore extract did not reduce TPN. Thus it appeared that transaldolase was not present in this preparation.

The enzyme reactions mentioned above may be summarized in the following diagram.



The results clearly show that phosphorylated hexose may be oxidized through this pathway when hydrogen acceptors are provided. More recent studies have shown that this oxidation seems to play a significant role in carbohydrate metabolism by wheat stem rust since the specific activity of carbon dioxide produced by uredospores of wheat rust in presence of glucose-1-C^{14} was significantly higher than with glucose labelled on any other position (9) and a similar result was obtained with an infected wheat plant by Shaw and Samborski (8).

Acknowledgment

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INFECTIOUS HEPATITIS AND THE CYTOLOGICAL RESPONSE IN EMBRYONATED EGGS[1]

By B. K. Buchner, R. C. French, F. Deinhardt,[2] J. C. Sinclair, Elizabeth McKee, and C. E. van Rooyen[3]

Abstract

The histiocyte response in embryonated eggs, following inoculation with acute infectious hepatitis serum, has been investigated and found to be both erratic and non-specific in character. High histiocyte counts were obtained with normal human serum, with serum from a case of simple obstructive jaundice, and with sera from convalescent cases of infectious hepatitis. Irrespective of the original source of inoculated material, it was impossible to transmit the histiocyte response by serial propagation in embryonated eggs. Consistently high histiocyte counts were obtained when the egg adapted strains of influenza and mumps viruses were employed. Moreover with influenza virus, the effect could be neutralized by specific influenza antiserum. Attempts to produce a similar histiocyte response with poliomyelitis virus types 1, 11, and 111 failed. In interpreting our results we have concluded that the histiocyte effect may be produced not only by certain egg adapted viruses but also by reactive components of human serum occurring in both normal and pathological states.

Introduction

Pollard and Diserens (1) claimed that when sera derived from cases of infectious hepatitis during the acute phase were introduced into the allantoic cavity of embryonated eggs, the histiocyte content of that fluid increased. Likewise the same workers considered that the histiocyte producing factor could be propagated serially in chick embryos and could be neutralized by sera from convalescent cases of infectious hepatitis or by gamma globulin. Previous heating of positive sera was shown to abolish the histiocyte producing effect. It was, furthermore, claimed that sera from cases of obstructive jaundice failed to produce histiocytosis. Experiments performed under similar conditions revealed that mumps and influenza viruses yielded the same results.

Experimental

We have attempted to repeat the work of Pollard and Diserens (1) with emphasis on two aspects, namely; (*a*) to determine whether the method described was applicable to the identification of sera from cases of acute infectious hepatitis and (*b*) to explore the use of this principle in the egg embryo cultivation of egg adapted and unadapted strains of viruses.

Materials

Sera were gathered from cases of infectious hepatitis occurring in the Province of Ontario and Metropolitan Toronto. These were obtained either two or three days prior to the onset of jaundice or within four days thereafter.

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Tests were also performed with sera taken during the acute phase from the well known Akiba experiments conducted by Dr. Werner Henle of the Children's Hospital, Philadelphia. Control sera employed in our series consisted of sera obtained from normal human blood donors in Philadelphia and Toronto and also a specimen of serum from a case of obstructive jaundice.

A strain of influenza virus (PR8) and a strain of mumps virus adapted to growth in the allantoic cavity (Barnes 8) were employed. Tissue culture strains of poliomyelitis viruses (Mahoney, MEF_1, and Saukett) were likewise subjected to the same methods of examination.

Methods

Procedures identical to those employed by Pollard and Diserens (1) were used in our experiments. These comprised the use of seven- to nine-day-old embryonated eggs, similar techniques of inoculation, the same dosage, the

TABLE I

HISTIOCYTE COUNTS* OBTAINED IN REPEATED TESTS ON VARIOUS SERA, NUTRIENT BROTH, AND SALINE

Origin	Sample Number	Inoculation Number						
		1	2	3	4	5	6	7
Nutrient broth		8						
Saline		16	4	0	11			
Saline (P+S)†		23†						
Normal serum O.D.H.	A	55	116	31				
C.R.C.	55521	39	24	35	7	45	7	14
C.R.C.	55522	40	31	108	0	9	147	
C.R.C.	55523	26	37	35	4	49	3	30
C.R.C.	55604	29	107	96	32	0	79	
C.R.C.	55605	53	103	71	39			
Phila	P3138	21	19	10				
Phila	P3134	55	80					
Phila	P3143	24						
Phila	P3132	35	45					
Phila	P3133	38	10	47				
Phila	P3126	40	200					
Phila	P2998	7						
Phila	P3028	16	34					
Phila	P3141	34	45					
Phila	P3123	228	9	166				
Infectious hepatitis Acute serum	DOB	177	146					
	EMM	14	90					
	LUB	8						
	DOW	15						
	GRAH	29						
	HOW	280	384	294				
	VAN	3	24					
	BAK	80	55					
	CUR	8						
	GAU	18						
	Akiba 3	33						
	" A2	91						
	" 5	24						
	" 2	82						
	" (6, A13)	362						
	" (A20, A16)	116						
Convalescent serum	CUR	15	10					
	LUB	24						
	DOB	59						
	HOW	127						
Obstructive jaundice serum	MUR	256	116					

NOTE. *In Tables I to V:*
**The figures recorded indicate the number of histiocytes present per 100 fields per stained slide.*
†P—penicillin 500 units and S—streptomycin 500 μgm. added.

same temperature and duration of incubation (35°C. for two to six days), the same method of harvesting, the preparation of stained smears, and the counting of histiocytes. Unstained material was also examined under the phase contrast microscope in order to provide an additional means for estimating the histiocyte response.

Results

Altogether 25 tests were performed with material from 22 typical cases of acute infectious hepatitis. Fifty-eight control tests were done using 16 normal sera and the serum from a case of obstructive jaundice. The effect of inoculating nutrient broth and saline was also studied. Each sample was investigated by inoculating a group of six eggs. The histiocyte counts obtained are recorded in Table I. The results show that the histiocyte response was erratic in behavior and failed to yield consistent values in consecutive tests with individual sera. Normal sera produced counts similar to those obtained with acute sera; namely 88% of normal and 72% of acute sera yielded counts of less than 100.

One infectious hepatitis serum (HOW) produced high counts on three consecutive tests. However, the sample of serum from the same patient (HOW) withdrawn during convalescence six weeks after the onset of illness likewise yielded a high count. Another acute serum (DOB) demonstrated high values twice, but when this serum was diluted 1:4 for a neutralization test, no histiocyte response occurred and thus the original result could not be repeated. Two Akiba pools showed high results, but insufficient sera made it impossible to repeat the tests. Since it was not possible to obtain consistent results on successive tests with individual samples of sera, further neutralization tests were not attempted.

Table II shows the histiocyte values obtained following the serial passage of normal, acute, and convalescent sera. Altogether 55 serial transfers of acute sera were attempted but in only three did the values exceed 100. In two of these samples the corresponding cell count performed with the phase contrast microscope did not confirm the high result found in the stained preparation, and with none of the samples could the effect be demonstrated in a subsequent egg passage.

Further experiments indicated that inoculation of saline, nutrient broth, or faecal extracts yielded lower values than those elicited with sera. Also it was noted that the addition of 500 units of penicillin and 500 μgm. of streptomycin tended to increase the histiocyte count.

Experiments with Known Viruses

Influenza

The PR8 strain of influenza virus was inoculated into the allantoic cavity of nine-day-old embryonated eggs, incubated at 35°C. for 48 hr., the allantoic fluids were aspirated, and the histiocyte cells enumerated. The results showed

TABLE II

HISTIOCYTE COUNTS* FOLLOWING SERIAL EGG PASSAGES OF VARIOUS SERA AND SALINE

Origin	Sample	Passage Number‡					
		Initial	2	3	4	5	6
Saline		23†	7	4			
Normal serum	55523	49	2	6			
Infectious hepatitis Acute serum	Pool J	105	0	15	15	13	15
	HOW	384	8(18)(9)‡	14	36	40	5
	DOB	146	8	3	27	7	
	EMM	90	<20	<20	41	3	
	BAK	80	17	39†	50	3	
	VAN	24	9(9)	182	3(65)		
	GRAY	5	7	6	38	0	
	Akiba 3	33	18	1	19	7	
	" A2	91	<20	68			
	" 5	24	164(180)	10(9)	6	6	
	" 2	82	12	0	5	25	
	" (6, A13)	362	27	23	3		
	" (A20, A16)	116	1	5			
	HUT	Not tested	12				
	GRAH	29	20				
	KROL	1	8				
Convalescent serum	TUC	17	15				
	VAN	135	31(14)				
	BAK	78	12(5)				
	HOW	127					
	GAUT	3					
	KROL	3					

‡*Figures in parentheses under passage number columns indicate histiocyte count obtained after repeated inoculations.*

TABLE III

HISTIOCYTE COUNTS* FOLLOWING INOCULATIONS OF INFLUENZA VIRUS

Inoculation	Dilution in saline	Result
PR8	1×10^{-2}	182
Saline	—	0
PR8	1×10^{0}	640
	1×10^{-2}	95
Saline	—	11
PR8	1×10^{-1}	526
	1×10^{-3}	199
	1×10^{-6}	42
	1×10^{-7}	30
PR8	1×10^{0}	150
PR8 + chick immune serum (1.S.)	1×10^{7}+1.S.	13

that high values up to 640 per 100 fields could be obtained with regularity by this procedure (See Table III). Furthermore the response could be neutralized by specific PR8 influenza antiserum.

Mumps

The Barnes 8 allantoically adapted strain of mumps virus was used for the tests. Seven-day-old embryos were inoculated, incubated for five days, and the fluid was harvested and examined for histiocytes as previously. The results obtained are recorded in Table IV and are similar to those obtained with influenza virus. Thus, the histiocyte response produced by the egg adapted strains of these two viruses has been confirmed.

Poliomyelitis Viruses

Three types of virus were used for the tests, Mahoney, MEF_1, and Saukett. They had been cultivated in monkey kidney tissue culture and possessed cytopathogenic doses (50% end point) of $10^{-7.3}$, $10^{-7.3}$, and $10^{-6.9}$ per 0.2 ml. respectively. The material was inoculated into the allantoic cavity of seven-day-old chick embryos, incubated for four days, the allantoic fluid harvested, and the histiocyte cells enumerated. No response was observed, and serial passages of allantoic fluids from inoculated eggs were likewise negative for histiocytes.

TABLE IV

HISTIOCYTE COUNTS* FOLLOWING INOCULATIONS OF MUMPS VIRUS

Inoculation	Dilution in saline	Results
Barnes (B8)	1×10^{-1}	491
	1×10^{-3}	104
	1×10^{-5}	92
	1×10^{-7}	33
	1×10^{-9}	23

TABLE V

HISTIOCYTE COUNTS* FOLLOWING INOCULATIONS AND ATTEMPTED PASSAGES OF POLIOMYELITIS VIRUS

Material	Dilution in saline	Passage Number		
		Initial	2	3
MEF_1	1×10^{0}	4	3	3
	1×10^{-1}	11	3	
Saukett	1×10^{0}	4	4	15
	1×10^{-1}	8		
Mahoney	1×10^{0}	12	5	5
	1×10^{-1}	5		
Nutrient medium	1×10^{0}	5	7	5
	1×10^{-1}	1		

Further Experiments

As a matter of interest we inoculated three samples of acute infectious hepatitis sera in the anterior chamber of a number of rabbits' eyes. The aqueous humor was aspirated three to four days later and examined for the presence of histiocytes. No significant differences could be detected between the cellular reactions produced by acute infectious hepatitis or normal sera. In another experiment an effort was made to determine whether the histiocyte response in eggs could be evoked with faecal extracts from cases of acute infectious hepatitis but again the results were negative.

Discussion

We have been able to confirm the work of Pollard and Diserens which demonstrated that a histiocyte response followed inoculation of embryonated eggs with influenza and mumps viruses. Tests with poliomyelitis viruses grown in monkey kidney tissue culture failed to evoke the same reaction. From this it was assumed that the histiocyte response could be elicited by a virus which had been adapted to egg cultivation. We have not been able to confirm the claims of Pollard and Diserens that sera from cases of acute infectious hepatitis will evoke a similar histiocyte response. Our experiments with sera from acute infectious hepatitis have suggested that the histiocyte response is no higher than that observed with various normal and other sera. On three occasions we encountered relatively high values with sera from three different cases of infectious hepatitis but it was not possible to reproduce this effect in serial passages. Neither was it possible to neutralize the effect with convalescent sera from cases of infectious hepatitis, and since it was impossible to obtain consistent results on successive tests with individual samples of sera, it is concluded that reliance could not be placed on the interpretation of neutralization tests.

In general, we have concluded that the histiocyte response is a reaction which may be produced by certain egg adapted strains of virus or by some non-specific irritant component in certain sera.

Acknowledgments

We have to thank Dr. Werner Henle of the Children's Hospital, Philadelphia, for the Akiba and mumps strains of viruses and our colleague, Dr. A. E. Franklin, for the poliomyelitis viruses. The work has been undertaken with the support of the Defence Research Board Grant No. 8915-04 for Studies on the Aetiology of Infectious Hepatitis.

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PROPAGATION OF INFECTIOUS BRONCHITIS VIRUS IN TISSUE CULTURE[1]

By J. E. Fahey and J. F. Crawley

Abstract

Two strains of infectious bronchitis virus were propagated serially in stationary flask cultures of chick embryo tissues. Both strains of virus could be grown in tube cultures of chick embryo tissues. Virus was produced by the cells for approximately one month without any evidence of damage to the cells. The strains tested could also be propagated in monkey kidney tissue cultures but not the L strain of cells. In monkey kidney cultures, virus was produced continuously for over one month without apparent damage to the cells.

Introduction

Infectious bronchitis of chickens is an acute, highly infectious respiratory disease caused by a virus (7). This disease is widespread throughout the North American Continent, and in Canada serological surveys have revealed that approximately 70% of the flocks are affected (3, 5). In Canada the disease is controlled by vaccination utilizing an attenuated strain of infectious bronchitis virus administered by the spray method (3, 4). In this work we have investigated the growth of infectious bronchitis virus in tissue culture with a view to determining what distinguishing characteristics, if any, it possesses under these circumstances.

The following avian viruses are considered to have been successfully propagated in tissue culture: fowl leucosis, fowl plague, Newcastle disease, psittacosis, Rous sarcoma, avian encephalomyelitis, and fowl pox (11). It is probable that a number of undiscovered viruses still exist among the avian species. It is felt that the discovery of such hypothetical viruses by tissue culture methods would be facilitated if knowledge were available concerning the nature of the effects of known avian viruses.

Material and Methods

The strains of infectious bronchitis virus used in this study were the Connaught Laboratories vaccine strain R (3) and the Beaudette embryo-passed strain (1). The choice of these two strains allows this study to be compared with other studies on the growth curves of the virus in chick embryos in which these two strains were also used (6).

Synthetic media were employed in our studies, with or without horse serum and chick embryo extract. The synthetic medium used was a modification of Medium 199 (10), and is referred to as Medium 597. This latter medium differs from Medium 199 in that the purines and pyrimidines have been omitted and that it contains Hank's balanced salt solution. Medium

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

597 was used only in studies involving serial passage of the virus in stationary flask cultures. In all other experiments Medium 597 was enriched by the addition of 20% horse serum and 2% chick embryo extract. The chick embryo extract was prepared by homogenizing decapitated 11-day-old embryos in a Waring blendor and resuspending the resulting embryo pulp in an equal volume of distilled water. Eggs from a flock not previously exposed to the virus were used in the preparation of the chick embryo extract. This eliminated the possibility of naturally-occurring infectious bronchitis virus antibodies. Non-immune flocks were selected by means of a serum-neutralization test (3).

The tissue for use in stationary flask cultures was obtained from 12–14-day-old embryonated eggs. The eggs were opened aseptically and the contents removed in such a manner as to leave the chorioallantoic membrane intact within the shell. The chorioallantoic membrane was then removed, washed three times in warm physiological saline, and minced finely with scissors. The resulting tissue fragments (0.5–1.5 mm.) were washed in Medium 597 and then dispensed into a 200 ml. Erlenmeyer flask containing 25 ml. of Medium 597. Each flask received a volume of tissue corresponding to approximately one-quarter of a chorioallantoic membrane. In those cases where liver tissue was used, the livers were removed from the embroyos, minced, treated as described above, and dispensed so that each flask received a volume of tissue corresponding to one chick embryo liver.

For stationary tube cultures, tissue from whole embryo and embryo heart was used. Tissue from whole embryo was prepared by mincing decapitated embryos with scissors as finely as possible and then forcing the embryo pulp through a No. 18 gauge needle mounted on a 20 ml. syringe. The tissue was washed twice by settling in three times its volume of Medium 597, and then 0.2 ml. was dispensed into $6 \times \frac{3}{4}$ in. test tubes placed in a rack at a 7° angle. The tubes were coated internally with formvar (0.4% formvar in ethylene dichloride) and after the tissue had adhered to the inside of the tube, 3.0 ml. of enriched Medium 597 was added. Formvar was used to facilitate anchorage of growing tissues to the wall of the test tube. The tubes were incubated at 37° C. for 5–10 days after which time the outgrowth of fibroblast-like cells was prominent. At this time the medium was changed and the cells used for infection experiments. The chick embryo heart fragments were treated in a similar way except that they were grown in uncoated tubes and were usually ready for use in 3–7 days.

The other cells utilized were Earle's *L* strain of cells (12) from the mouse and trypsinized monkey kidney cells. The *L* cells were maintained in Kolle flasks and subcultured as required. The monkey kidney cells were seeded into tubes or T-60 flasks and used after approximately 7–10 days' growth, at which time the medium was changed.

All virus titrations were performed in 9-day chick embryos with inoculations being made into the chorioallantoic sac. Tenfold dilutions were made of each suspension to be titrated and 10 eggs were inoculated with each

dilution. The eggs were candled daily after inoculation for a period of 10 days. Only dead embryos were considered in the estimation of the potency of the virus suspensions, all estimations of the LD_{50} were made by the Karber method (8).

Results

Stationary Flask Cultures

The first information desired in this study was whether the Beaudette strain of virus would multiply in tissue culture. To investigate this it seemed desirable to utilize a tissue known to support the growth of the virus in an intact organism, that is, chorioallantoic membrane. The cultures were infected with 0.5 ml. of chorioallantoic fluid having an LD_{50} of $10^{-4.5}$. The fluid was then harvested after 48 hr., the tissue particles being spun down by light centrifugation and passage continued by again transferring 0.5 ml. The fluid removed was titrated in eggs at each passage. Since the titers became quite high after the 3rd passage, subsequently 0.5 ml. of a 1/100 dilution was used from the 4th through to the 10th passage. The original virus was diluted greater than $1:10^{26}$ as a result of 10 serial transfers. The titers of the fluids harvested from each passage are shown in Table I.

TABLE I

TITRATION OF FLUIDS OBTAINED IN SERIAL PASSAGE OF INFECTIOUS BRONCHITIS VIRUS (BEAUDETTE) IN STATIONARY CULTURES OF CHORIOALLANTOIC MEMBRANE TISSUE

Passage No.	Log LD_{50}
1	−5.28
2	−6.30
3	−6.42
4	−5.62
5	−7.36
6	−7.82
7	−8.28
8	−8.11
9	−8.36
10	−7.47

Attempts to passage this strain in chick embryo liver or in whole embryo tissue fragments did not meet with the same success. The preliminary trials using embryonic liver provided conflicting results, the virus propagating in some cases, but not in others. It was eventually found that in those instances where the virus would not grow in the chick embryo liver, it was apparently due to the presence of antibodies in the egg. The livers of those embryos which possessed antibodies in their yolk sacs would not support the growth of the virus when a simple washing of the minced tissue was employed. However, 4–5 washings served to remove these antibodies and the tissue would then support growth, but at a rate considerably lower than that in the chorioallantoic membrane. The titer of virus passaged in embryonic liver did not ever exceed $10^{-3.5}$, but since the Beaudette strain was

passaged six times (representing a dilution of greater than $1:10^{12}$ of the initial stock) this served to establish that propagation did take place in this tissue. Essentially similar findings were obtained with whole embryo in tissue culture with titers tending to be somewhat higher, the maximum being $10^{-4.8}$.

The R strain was passaged six times in chorioallantoic tissue and the LD_{50} of the final passage was $10^{-5.1}$. The initial inoculum had an LD_{50} of $10^{-3.1}$ and passage was conducted by transferring a 1/50 dilution of the previous passage in each case. It was not possible to passage the R strain serially in embryonic liver or in whole embryo, even when the tissues were carefully washed or when eggs from a flock not exposed to infectious bronchitis virus were used as a source of tissue.

In both instances the virus strains were identified at the end of the tissue culture passage series by means of a neutralization test involving standard infectious bronchitis antiserum (3).

Growth Curves

Since growth of infectious bronchitis virus was shown to be supported in tissue culture, it appeared of interest to examine the time course of virus production. For this purpose the Beaudette strain was used at the sixth tissue culture passage in the growth curve studies. Fluid harvested from the sixth passage was diluted 1/100 and 0.5 ml. was added to each of three 200 ml. Erlenmeyer flasks containing minced chorioallantoic membrane in 25 ml. of enriched Medium 597. Aliquots of 0.4 ml. were removed periodically from each flask, pooled, and titrated in eggs. A similar procedure was used with the R strain at the third passage level. The results of these growth curve studies are shown graphically in Fig. 1.

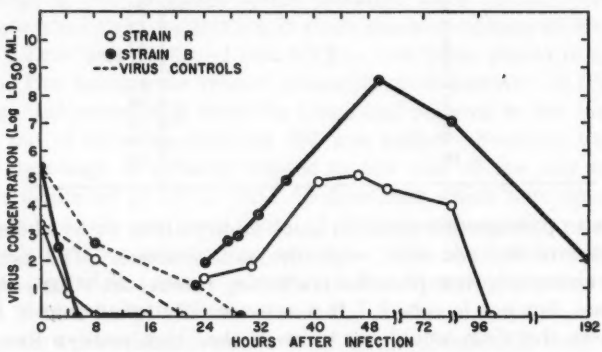

FIG. 1. Growth of infectious bronchitis virus in tissue culture.

It can be seen from Fig. 1 that a suspension of either strain of virus in the medium alone results in complete inactivation within 30 hr. In both instances the adsorption period was from four to eight hours and after this period the virus was no longer demonstrable. The latent period for the Beaudette strain in three experiments varied from 12 to 18 hr. and for the

R strain from 20 to 26 hr. It is apparent that the technique employed did not detect the first virus appearing in the new cycle since the aliquots were removed at wide intervals. Possibly the newly formed virus was adsorbed by uninfected cells and the appearance of the first virus after the latent period represents an amount of virus in excess of that required to saturate all cellular adsorptive sites.

The strains differed in other respects, such as the rate of disappearance of active virus and the final titer reached. Since the tissue was not actively metabolizing (no change in pH of control or infected cultures), it might be expected that active virus production would cease after a few days. However, it is apparent from Fig. 1 that this is not the case, especially for the Beaudette strain. That portion of the curve after 48 hr. might be considered as the result of an equilibrium state between release of new virus and inactivation of existing virus particles. It appears that new virus is being formed for at least eight days for the Beaudette strain and at least three days for the R strain.

It is of some interest to note that the maximum titers obtained in tissue culture were reached in approximately 40 hr. for the R strain and in about 48 hr. for the Beaudette strain. In chick embryos the maximum titer is reached at 24–30 hr. for the R strain and in 12 hr. for the Beaudette strain (6).

Stationary Tube Cultures

Cultures of whole chick embryo or chick embryo heart were prepared as previously described, allowed to grow out, and infected with either the R or the Beaudette strain. The fluids were changed and titrated at intervals, in each case the fluid from three tubes being pooled for a titration. The results of one experiment in which whole embryo tissue was used are shown in Table II. In this experiment 0.1 ml. of a 1/100 dilution of chorioallantoic fluid (Beaudette strain) with an LD_{50} of $10^{-3.2}$ was added to the tubes.

It was found in these experiments that the pH of medium in infected cells did not change at a rate appreciably different to that of uninfected controls.

TABLE II

GROWTH OF INFECTIOUS BRONCHITIS VIRUS IN STATIONARY TUBE CULTURES OF WHOLE EMBRYO

Interval after infection (days)	Log LD_{50} of tissue culture fluids	
	R strain	Beaudette strain
1	−2.3	−3.6
2	−2.6	−4.6
6	−2.5	−4.1
8	−3.1	−5.3
10	−4.8	−5.8
13	−2.2	−4.7
17	−2.8	−5.4
20	−4.1	−5.1

Furthermore, daily microscopic examination of the cell layer failed to reveal any evidence of a cytopathogenic effect. The production of virus ceased only when the cell layer began to peel from the glass and this destruction of the cell layer occurred in the control cultures at the same time as in the infected culture. Thus, experiments, such as cited in Table II, indicated that infected chick embryo cells will continue to produce virus over a long interval of time without any apparent effect on the cells.

Essentially the same observations were made in cultures of chick embryo heart fibroblasts; these cells supported growth of the virus as long as the outgrowth remained intact. Similarly no cytopathogenic effect or difference in rate of change of pH from the controls could be observed in these cultures.

Growth in Other Animal Cells

Freshly trypsinized monkey kidney cells were dispensed in T-60 culture flasks containing 10 ml. of enriched Medium 597. After 7–10 days, the medium was changed and 1 ml. of chorioallantoic fluid with an LD_{50} of $10^{-4.1}$ was added to each flask, controls excepted. The fluid was removed entirely periodically and the cell layer rinsed once and the medium replaced. The results of a series of titrations of fluids removed in this manner are shown in Table III.

TABLE III

GROWTH OF INFECTIOUS BRONCHITIS VIRUS (BEAUDETTE) IN MONKEY KIDNEY CELLS

Interval after infection (days)	Log LD_{50}
2	−2.9
8	−3.3
19	−3.2
22	−4.8
23	−3.2
26	−3.8
30	−3.7

In these experiments it was also found that the pH of infected cultures did not differ from those of control or uninfected cultures. There was also no evidence of any cytopathogenic effect at any time in these experiments. Thus one can also conclude from these experiments that infectious bronchitis virus will propagate for prolonged periods in monkey kidney cells without any apparent damage to the cells. The R strain could also be propagated for three to four weeks in monkey kidney cells in a similar manner, although the maximum titer never exceeded $10^{-2.3}$.

The above experiments showed that these viruses would propagate in monkey kidney cells. In the next experiment the classical cycle of multiplication was examined. To this end, monkey kidney cells growing in stationary

tube cultures were infected with chorioallantoic fluid with an LD_{50} of $10^{-4.2}$ (0.1 ml. into 3 ml. of medium) and aliquots removed periodically thereafter. It was found that the virus began to disappear within one hour and was all gone within eight hours. It should be remembered that under these conditions the virus is relatively stable for approximately 24 hr. No fresh virus then reappeared for about 8–10 hr., and within 24 hr. after the first appearance of the new virus the LD_{50} of harvested fluid rose to $10^{-6.5}$ and remained at this high level for approximately one week; thereafter it declined gradually, and finally disappeared about three weeks after initial infection. This coincides with the time that the cells degenerate in the control tubes. The virus harvested from both flask and tube cultures of monkey kidney cells was identified by means of a serum–neutralization test. Since uninfected cultures were similarly harvested and used as control inoculations it was proved that the virus being produced by the monkey kidney cells was indeed infectious bronchitis virus and not a latent monkey virus.

Attempts were also made to determine if growth of infectious bronchitis virus could occur in L cells. All attempts proved fruitless. There was no adsorption of virus, no cytopathogenic effect, nor could the virus be passaged serially in these cells.

Discussion

The finding that infectious bronchitis virus could be propagated in tissue culture preparations of chick embryo tissues was not unexpected since these tissues constitute a natural habitat. However, it is of interest that this virus could be propagated in monkey kidney cells. The fact that no significant cytopathogenic effect was found in any of the tissues tested indicates some obvious difficulties in future studies of this virus. Nevertheless, the absence of a cytopathogenic effect during a period of release of large amounts of virus from the cells may be of greater interest in other respects.

It is known that infectious bronchitis virus persists in many instances on certain premises where a continuous program of growing stock is maintained. The finding that this virus, once it has infected a culture, will continue to be produced for a long period without visibly affecting the cells raises the question of whether cells in an intact animal might not behave in an analogous fashion. Production of virus at an extremely low level in a recovered flock might easily serve to perpetuate the infection in nature.

The mechanism responsible for this physiological situation in infected cultures is of course obscure at this time and will require a more refined analysis. However, in some respects this situation resembles that observed in lysogenic bacteria which are able to produce virus over an indefinite number of generations (2, 9).

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OBSERVATIONS ON THE CULTURE AND MAINTENANCE OF A FREE-LIVING AMOEBA[1]

By A. Bakerspigel[2]

Abstract

A free-living amoeba isolated from creek water could be grown at 24° C. on a solid malt agar medium together with a pure culture of a motile Gram-negative bacillus, isolated from the same sample of water. The bacilli apparently served as the sole source of food for the trophozoites, the malt in the medium being utilized by the bacilli for their own growth. In addition, the encysted form has been maintained on this medium at 24° C. for the past 12 months and at 4° C. for 10 months. Transfers to fresh medium of cysts from such cultures yielded numerous trophozoites after 12 hr. incubation at 24° C., provided that metabolically active bacilli were present.

Introduction

A number of recent reports (1–4, 6–9) have described the difficulties encountered in satisfactorily cultivating and maintaining pure cultures of amoebae, such as *Endamoeba histolytica*. One of the major difficulties has been to maintain the amoebae for long periods in or on a synthetic medium in the absence of rice grains, bacteria, or protozoa. This difficulty has also been encountered in culturing and maintaining free-living species, such as *Amoeba proteus*. For example, Prescott and James recently reported (5) that *A. proteus* could be cultured on an inorganic medium and fed exclusively on *Tetrahymena*, but no particular effort was made to exclude bacteria from these cultures.

A similar problem arose in this laboratory when a reliable and simple method was sought for culturing and maintaining an unidentified species of amoeba living in creek water. After various media and procedures were tested, a method was employed which has produced satisfactory results. This method, which may be adapted for use in other laboratories, has provided a reliable and constant source of trophozoites and cysts useful for demonstration and cytological purposes.

Materials and Methods

Culture Medium

Malt agar medium (Difco No. 0024-01), to which an extra 5 gm. of agar per liter was added, was heated just to the point of boiling and dispensed in 10 ml. amounts in 25 ml. screw-capped tubes. The medium was then sterilized in the autoclave at 15 lb. pressure for 15 min. and the tubes slanted. The final pH of the medium was 5.5. Screw-capped tubes were found to be more useful than those with cotton plugs since the medium did not dry out so quickly and contamination of cultures with other bacteria or fungi was easily avoided.

[1] *Manuscript received April 25, 1956.*
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Source of Food for Trophozoites

Inoculum from a pure culture of a motile Gram-negative bacillus, isolated from the same sample of water as the cysts, was added to the medium as a source of food for the trophozoites.

Initiation of Cultures

Bacteria-free cysts were obtained by pipetting 15 ml. of the creek water into each of six test tubes in which an aureomycin-impregnated disk (10 μgm.) had been placed. After four to six hours at 24° C. each tube was centrifuged at 1000 r.p.m. for five minutes to concentrate the cysts, which were then washed three times in sterile distilled water. Cysts were then checked for the presence of live bacteria by inoculation on a malt agar slant which was incubated at 24° C. for three days.

Cultures were initiated by pipetting a few drops of water containing bacteria-free cysts onto a malt agar slant. A small loopful of a 24 hr. culture of the bacilli grown on malt medium was then spread over the surface of each slant. Inoculated tubes were incubated at 24° C. in a slanted position so that the whole surface of the medium was kept moist during the first 48–72 hr. incubation. The caps were then tightened and the tubes were stored in an upright position either at 24° C. or in the refrigerator at 4° C. Subcultures were made by inoculating fresh malt agar slants with trophozoites and/or cysts from the initial cultures.

Observations

Numerous trophozoites were obtained after 12 hr. at 24° C. when the cysts and bacilli were inoculated together on the malt agar medium. Usually, after 10–14 days, many amoebae encysted and it was in this form that they were maintained for long periods. For example, Fig. 1 shows cysts (10.8–18μ in diameter) from a culture (Fig. 2) maintained at 24° C. for the past 12 months. Transfers of such cysts to fresh malt agar yielded trophozoites within 12 hr. Similarly, cysts taken from cultures stored at 4° C. for 10 months also yielded trophozoites after 12 hr. incubation at 24° C. In all cases large numbers of motile bacilli were obtained when subcultures were made from tubes stored at either temperature.

These results show that this free-living amoeba can be cultured on a solid malt agar medium and also maintained on it in the cyst form for at least 10–12 months. However, the motile bacilli had to be present in the culture, not only for mass excystation to occur, but also as food for the trophozoites. If heat- or antibiotic-killed bacilli were added no excystation occurred either on the malt agar or on any of the other media tested. Moreover, as soon as most of the bacilli lost their motility, which usually occurred five to seven days after inoculation on a slant, the number of cysts in the culture increased. These observations suggested that the motile bacilli utilized the malt for their own needs at the same time elaborating one or more factors necessary for excystation. However, results from experiments showed that such was not

the case. Thus, when aliquots of liquid malt medium, in which the bacilli were grown for 24, 48, or 72 hr., were added to a plain agar slant inoculated with cysts, no excystation occurred. It was also possible that the bacilli eliminated one or more metabolic products which might be used by the trophozoites during growth and division. However, it was found that when a heavy suspension of motile bacilli was spread over sterile cellophane placed on plates of malt agar, the surfaces of which had first been seeded with cysts and a small number of trophozoites, no excystation occurred nor did the number of trophozoites increase. In fact, microscopic examination showed that after 48 hr. very few trophozoites could be found on the inoculated areas of the medium. Nevertheless, the possibility of such products being produced by the bacilli cannot be overlooked and further nutritional studies may or may not reveal them to be present.

Admittedly the most favorable and perhaps dependable method for culturing and maintaining free-living and other amoebae would be in or on a simple, chemically defined medium. However, in the absence of such an ideal medium, the method described above was found to be satisfactory for obtaining large numbers of trophozoites and cysts. The presence of bacilli in these cultures did not interfere with the demonstration of cysts or the movement of trophozoites. In fact, preparations from such cultures aided students in appreciating the complexity of microbial populations and the interrelationship of microorganisms in nature.

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MICROCOCCUS CEROLYTICUS, NOV. SP., AN AEROBIC LIPOLYTIC ORGANISM ISOLATED FROM THE AFRICAN HONEY-GUIDE[1]

By H. Friedmann[2] and J. Kern

Abstract

A micrococcus has been isolated from the droppings and intestinal contents of the African honey-guide. Because of its marked cerolytic activity, the organism has been named *Micrococcus cerolyticus*, nov. sp. A culture of this organism is being deposited at the American Type Culture Collection, Washington, D.C.

In our investigations on the metabolism of beeswax by an African bird, *Indicator minor*, commonly known as the lesser honey-guide, a micrococcus was routinely isolated which was subsequently implicated in the digestion of beeswax by the bird. Detailed studies dealing with the mechanism of wax degradation by the honey-guide and the role of this organism in this process have been reported elsewhere by Friedmann and Kern (3). The unusual characteristic of this micrococcus to metabolize wax prompted a detailed study of the organism itself which is reported herein. Because of its dominant "cerolytic" activity it is proposed to name this organism *Micrococcus cerolyticus*.

The organism was consistently isolated in large numbers from all honey-guides received from Africa; the primary isolation was made by streaking suspensions of aseptically collected droppings or of the intestinal contents of sacrificed birds on trypticase soy (BBL), veal infusion (Difco), and blood agar plates, with incubation at 30° and 37° C. Transfers from colonies to common laboratory media yielded excellent growth. The optimum temperature for the growth of the organism was found to be between 37° and 40° C.; good growth was also observed at 30° C. but not at 50° C. Cultures were maintained on fresh moist slants of trypticase soy agar with incubation at 37° C., and for all subsequent determinative work were 16–20 hr. old.

The coccus is Gram-positive, non-motile, and has an average diameter of 0.5 μ to 0.75 μ in 16–18 hr. cultures when grown on trypticase soy agar slants; it appears generally in irregular masses when observed in stained preparations. Retention of the Gram-positive staining reaction is noted for periods up to 48 hr. after which time the cells begin to assume Gram-negative characteristics. In 18–24 hr., colonies on trypticase soy and nutrient gelatin agar plates are small, raised, opaque, and entire with a grayish-white pigment which becomes more glistening after 48 hr. Growth after 24 hr. in nutrient broth is heavy with no pellicle and a heavy sediment, which may be partially dispersed upon shaking. The growth on slants of veal infusion or trypticase soy agar after 24 hr. at 37° C. is flat, spreading, and grayish-white, and it is revealed as having a creamy non-elastic consistency when touched with an inoculating needle.

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Fermentation of glucose, lactose, and sucrose with acid formation occurs in 24 hr.; no gas is produced from glucose in five days. Acid is not formed from maltose, mannitol, sorbitol, or glycerol although salicin is fermented in 48 hr.

All cultures give a negative reaction for indol and acetylmethylcarbinol; starch and gelatin are not hydrolyzed. The latter was studied by the ordinary stab procedure in 12% gelatin and by the nutrient agar- 0.4% gelatin plate method, while conventional starch agar plates were employed for the former determination. Nitrate is reduced rapidly as evidenced by a positive reaction in 18 hr.; the test for catalase is positive. Urea and ammonium phosphate are not utilized as sources of nitrogen; the media are those recommended in Bergey's Manual (1). Hydrogen sulphide is not produced while litmus milk becomes acid in 24 hr. and in three days is coagulated with concurrent reduction of the indicator.

The organism shows *beta*-hemolysis on sheep blood agar in 18 hr. at 37° C. but not at 30° C. until 48 hr. have elapsed. Complete clearing occurs in 72 hr. at the former temperature. The coagulase test is negative.

Two methods were employed to demonstrate the lipolytic activity of the organism. When the nile blue – triglyceride – infusion agar medium described by Turner (5) was employed, deep blue zones were found to surround the growth of the organism after 18–20 hr. at a temperature of 37° C. This is contrasted to the pale blue color of the uninoculated medium. A recent method described by Bulder (2) was employed to confirm this biochemical activity. Droplets of sterile olive oil are sprayed on the surface of a nutrient agar plate immediately following the streaking of the test organism; aseptic precautions are taken. After a suitable incubation period, lipolysis is manifest by a change in the appearance of the oil droplets when the plate is examined with the low power microscope. It is found that after two days at a temperature of 37° C., the droplets in contact with or in the vicinity of the growth became granular, irregular in shape, and opaque, indicative of fat hydrolysis. The droplets on the uninoculated control plates are unchanged in appearance.

TABLE I

DIFFERENTIATION BETWEEN *Micrococcus cerolyticus* AND OTHER MICROCOCCI

Organism	Reactions on:			
	Maltose	Salicin	Glycerol	Litmus milk
M. cerolyticus	—	+	—	Acid; coag.; reduction
M. freundreichii	—	—	±	Acid; coag.; peptonization
M. citreus	+	±	±	Acid
M. epidermidis	+	—	+	Acid
M. conglomeratis	—	—	—	Acid

Resting-cell experiments revealed that this micrococcus can metabolize a variety of lipids, such as cottonseed oil, olive oil, tributyrin, and beeswax.

All of our efforts to find previous reference to this organism in a search of the literature have been unsuccessful. The micrococcus isolated by Toumanoff (4) from a foul brood of bees differs from this organism in several of the biochemical activities. Similarly, comparison with four other species of micrococci, including *M. epidermidis* obtained from the American Type Culture Collection, have indicated that the organism has not been classified previously (Table I).

The organism described in this paper has been deposited with the American Type Culture Collection.

The authors wish to express their appreciation to Dr. Samuel J. Ajl for his helpful suggestions and advice during the course of this study.

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